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(54) Monoclonal antibodies to human breast carcinoma cells and their use in diagnosis and therapy.

(57) Monoclonal antibodies to adenocarcinoma cells, and, in particular, breast carcinoma cells, are produced by a hybridoma formed by fusing mouse lymphocytes and mouse myeloma cells. The monoclonal antibodies are capable of shrinking solid tumors associated with human breast. The monoclonal antibodies, specifically, F36/22 monoclonal antibodies, can be used diagnostically and therapeutically.

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MONOCLONAL ANTIBODIES TO HUMAN BREAST CARCINOMA CELLS AND THEIR USE IN DIAGNOSIS AND THERAPY

1. INTRODUCTION

This invention relates to the production of and applications for monoclonal antibodies specific for tumor antigens. More particularly, this invention relates to monoclonal antibodies against cell surface antigenic determinants expressed maximally on breast carcinomas and also present on other adenocarcinomas. Monoclonal antibodies capable of reacting with cell-surface antigens are of value for the immunoclassification and detection of disease and represent novel agents for immunotherapy. The monoclonal antibodies of this invention possess distinctive characteristics and capabilities which make them suitable for *in vitro* clinical diagnostic purposes. Moreover, they are of great potential importance for *in vivo* tumor localization and cancer therapy in humans.

The monoclonal antibodies exhibit a high level of binding to breast carcinoma cells and are capable of experimental *in vivo* tumor localization. They bind to well-differentiated as well as to poorly-differentiated tumors. The antigen recognized is a cell surface component that remains unmodulated after exposure to antibody. The monoclonal antibodies exhibit the ability to fix human complement. Most importantly, the monoclonal antibodies of this invention are capable of inducing a rapid and significant volume reduction of established, progressively growing, solid human breast tumors xenografted in animals (mice). The tumoricidal effectiveness is quite remarkable with tumor volume shrinkage of greater than 70% in short intervals (three days) after administration of low doses (100 µg) of antibody. Immunotherapy occurs passively, independently of any cytotoxic compounds.

The invention provides methods for production of the monoclonal antibodies by hybridoma techniques. Once cloned, cell lines can be maintained continuously to produce an unlimited homogeneous monoclonal antibody population that can be isolated and/or purified and used clinically for in vitro immunohistological, immunocytological or immunoserological diagnosis, in vivo diagnosis by localization of tumors and metastases, and immunotherapy of human cancers, particularly those of the breast.

2. BACKGROUND OF THE INVENTION

2.1. MONOCLONAL ANTIBODIES

Kohler and Milstein are generally credited with having devised the techniques that successfully resulted in the formation of the first monoclonal antibody-producing hybridomas [G. Kohler and C. Milstein, Nature 256:495-497 (1975); Eur. J. Immunol. 6:511-519 (1976)]. By fusing antibody-forming cells (spleen lymphocytes) with myeloma cells (malignant cells of bone marrow primary tumors) they created a hybrid cell line, arising from a single fused cell hybrid (called a hybridoma or clone) which had inherited certain characteristics of both the lymphocytes and myeloma cell lines. Like the lymphocytes (taken from animals primed with sheep red blood cells as antigen), the hybridomas secreted a single type of immunoglobulin specific to the antigen; moreover, like the myeloma cells, the hybrid cells had the potential for indefinite cell division. The combination of these two features offered distinct advantages over conventional antisera. Whereas antisera derived from vaccinated animals are variable mixtures of polyclonal antibodies which never can be reproduced

identically, monoclonal antibodies are highly specific immunoglobulins of a single type. The single type of immunoglobulin secreted by a hybridoma is specific to one and only one antigenic determinant, or epitope, on the antigen, a complex molecule having a multiplicity of antigenic determinants. For instance, if the antigen is a protein, an antigenic determinant may be one of the many peptide sequences [generally 6-7 amino acids in length (M.Z. Atassi, Molec. Cell. Biochem. 32: 21-43 (1980))] within the entire protein molecule. Hence, monoclonal antibodies raised against a single antigen may be distinct from each other depending on the determinant that induced their formation; but for any given clone, all of the antibodies it produces are identical. Furthermore, the hybridoma cell line can be reproduced indefinitely, is easily propagated in vitro or in vivo, and yields monoclonal antibodies in extremely high concentration.

2.2. APPLICATION OF MONOCLONAL ANTIBODIES TO CANCER

Monoclonal antibodies presently are being applied by investigators to the diagnosis and treatment of cancer [For a general discussion of the topic, see Hybridomas in Cancer Diagnosis and Treatment, Mitchell, M.S. and Oettgen, H.F., (eds.), Progress in Cancer Research and Therapy, Vol. 21, Raven Press, New York (1982)]. It has been reported that monoclonal antibodies have been raised against tumor cells [U.S. Pat. No. 4,196,265], carcinoembryonic antigen [U.S. Pat. No. 4,349,528], and thymocytes, prothymocytes, monocytes and suppressor T cells [U.S. Pats. Nos. 4,364,933; 4,364,935; 4,364,934; 4,364,936; 4,364,937; and 4,364,932]. Recent reports have demonstrated the production of monoclonal antibodies with various degrees of specificity to several human malignancies, including mammary tumor cells [Colcher, D.

et al., Proc. Natl. Acad. Sci. U.S.A. 78:3199-3203 (1981)], lung cancers [Cuttitta, F., et al., Proc. Natl. Acad. Sci: U.S.A. 78:495-4595 (1981)] malignant melanoma [Dippold, W.G. et al., Proc. Natl. Acad. Sci. U.S.A., 77:6114-6118 (1980)], colorectal carcinoma [Herlyn, M. et al., Proc. Natl. Acad. Sci. U.S.A. 76:1438-1442 (1979)], lymphoma [Nadler, L.M. et al., J. Immunol. 125:570-577 (1980)], and neuroectodermal tumors [Wikstrand, C.J. and Bigner, D.C., Cancer Res., 42:267-275 (1982].

Investigators have noted the potential immunotherapeutic value of monoclonal antibodies and some investigators have investigated therapeutic efficacy in both animal and human subjects [Miller, R.A. et al., New Eng. J. Med. 306:517-522 (1982); Ritz, J. and Schlossman, S., Blood, 59:1-11 (1982); and Kirch, M.E. and Ulrich, H. J. Immunol, 127:805-810 (1981)]. Although most studies have described the effects of cytotoxic drug-antibody conjugates, [Beverly, P.C.L., Nature, 297:358-9 (1982); Krolick, K.A. et al., Nature, 295:604-5 (1982); Krolick, K.A. et al., Proc. Natl. Acad. Sci. U.S.A., 77:5419-23 (1980); Arnon, R. and Sela, M., Immunol. Rev., 62:5-27 (1982); Raso, V. et al., Cancer Res., 42:457-64 (1982); and DeWeger, R.A. and Dullens, H.F.J., Immunol. Rev. 62:29-45 (1982)], experimental and clinical studies on passive immunotherapies have been attempted [Sears, H.F. et al., Lancet, i:762-65 (1982); Rosenberg, S.A. and Terry, W.D., Cancer Res., 25:323-88 (1977); Herlyn, D.M. et al., Cancer Res., 40:717-21 (1980); Scheinberg, D.A. and Strand, M., Cancer Res. 42:44-9 (1982); Koprowski, H. et al., Proc. Natl. Acad. Sci. U.S.A., 75:3405-9 (1978); and Young, Jr., W.W. and Hakomori, S.-I., Science, 211:487-9 (1981)]. In the experimental setting, however, most studies have dealt with the concurrent administration of monoclonal antibody and tumor inoculum, or

administration within several days of the implantation of tumor cells, resulting in a decreased tumor take or growth rate of xenografts. In this context, these data form a basis for the immunoprophylaxis of tumor development. It is an object of the present invention to provide monoclonal antibodies demonstrating a therapeutic effect on progressively-growing established tumors. To our knowledge, prior to this invention, tumor volume reduction against well established progressively growing solid tumors with the use of passive monoclonal antibody has not been reported.

2.3 MONOCLONAL ANTIBODIES TO MAMMARY CELLS

Several investigators have reported on the production of monoclonal antibodies against epitopes of various normal and malignant mammary cell components. [Arklie, J., et al., Int. J. Cancer, 28:23-29(1981); Ciocca, D.R., et al., Cancer Res., 42:4256-4258(1982); Colcher, D., et al., Proc. Natl. Acad. Sci., U.S.A. 78:3199-3203 (1981); Foster, C.S., et al., Virchows Arch. Pathol. Anat., 394:279-293(1982); Greene, G.L., et al., Proc. Natl. Acad. Sci. U.S.A., 77:5115-5119(1980)]; McGee, J.O'D., et al., Lancet 2:7-11(1982); Nuti, M. et al., Int. J. Cancer, 29 :539-545(1982); and Taylor-Papadimitriou, J., et al., Int J. Cancer, 28:17-21(1981)]. Many of the antigens recognized above are differentiation-related; therefore these antibodies are most suited to histologically assess the differentiated status or grade of tumor specimens. For example, monoclonal antibodies directed against several antigens of human milk-fat-globule membranes have been produced. These antibodies have proven useful in studying the derivation of cell cultures, in evaluating the phenotypic expression of antigens in neoplastic transformation, and have served

as differentiation markers in breast cancer, and as immunodiagnostic reagents in the quantitation of antigens in the sera of breast cancer patients [Arklie, J., et al., Int. J. Cancer, 28:23-29(1981); Ceriani, R.L., et al., Proc. Natl. Acad. Sci. U.S.A., 74:582-586(1977); Ceriani, R.L., et al., Proc. Natl. Acad. Sci., 79:5420-5424(1982); Foster, C.S., et al., Virchows Arch. Pathol. Anat., 394:279-293(1982); and Taylor-Papadimitriou, J., et al., Int J. Cancer, 28:17-21(1981)];. However, there is further need for monoclonal antibodies which are differentiation-unrelated and of use to detect antigen occurring in poorly-differentiated breast tumors. It is an object of this invention to provide such monoclonal antibodies. Such reagents may reflect subtle biochemical or antigenic differences in breast cancer, independent of tumor grade. The use of such antibodies can add significant information regarding functional classificationss of individual breast tumors to augment clinical classifications.

The pattern of staining for the monoclonal antibody of this invention is distinct from the reactivities of previously described monoclonal antibodies which recognize antigens expressed by breast tumors. Arklie et al. [supra] have described monoclonal antibodies directed against human milk-fat-globule membranes. These antibodies showed a stronger staining reaction with well-differentiated (grade I) ductal carcinomas than undifferentiated (grade III) tumors. By comparison, the monoclonal antibody provided herein stained poorly-differentiated tumors equally well as well-differentiated breast carcinoma specimens. Therefore, the monoclonal antibody identifies a sub-class of breast carcinomas which is histologically indistinguishable from tumors lacking antigen expression.

Nuti et al. [supra] have produced monoclonal antibodies against human metastatic breast carcinoma cells which have been used to indicate tumor antigen heterogeneity, but which failed to react with normal and benign mammary tissues or uterus. Other reported monoclonal antibodies directed against carcinoma-associated antigens [McGee et al., supra] also did not react with any benign conditions of the breast and very few normal tissues. Foster et al. [supra] have also reported the production of monoclonal antibodies which were used to show significant heterogeneity of antigen expression within breast tumors, but whose specifities are distinct from the monoclonal antibodies of the present invention.

3. SUMMARY OF THE INVENTION

Prior to the present invention, applicants believe there has been no report of a clinically useful preparation of monoclonal antibodies which, upon passive administration, is capable of reducing the volume of well established, progressively growing solid tumors associated with the human breast. In fact, it is applicants' belief that volume reduction of any other established and growing solid tumors with the use of passive monoclonal antibody immunotherapy has not been reported. The present invention provides methods and compositions for producing novel monoclonal anti-breast carcinoma antibodies with specific binding, cytotoxic and tumor shrinkage capabilities and encompasses the use of said antibodies for cancer immunodiagnosis and passive immunotherapy in humans.

Specifically, the invention provides novel hybridoma-derived monoclonal antibodies which react with a determinant (epitope) located both on differentiated

mammary duct epithelia and on certain breast carcinomas; the monoclonal antibodies do not react with estrophilin, bone marrow stem cells or lymphoid cells. In contrast to the expression of previously described differentiation antigens, the epitope recognized by the monoclonal antibodies of this invention is also found in a high percentage of poorly-differentiated carcinomas of the human breast. The extent of antigenic expression, as detected by the monoclonal antibodies, is similar for well and poorly differentiated tumors and is independent of the histological characteristics of the tumor. The monoclonal antibodies can be used immunologically to sub-classify tumors which are histologically indistinguishable by conventional histopathological staining techniques and to establish phenotyping of breast cancer. Hence, the monoclonal antibodies of the present invention represent new in vitro immunodiagnostic reagents for the early and accurate detection of certain cancers.

The epitope recognized by the monoclonal antibody of this invention is also present on other adenocarcinomas, including those of the colon, ovary, uterus, pancreas and prostate (see Section 5.8.1), although the antigenic determinant is expressed at a lower level than in breast cancer. The normal tissue of these histotypes (colon, ovary, uterus, pancreas, prostate, etc.) contain no detectable levels of the determinant. Therefore, the expression of the antigen recognized by the monoclonal antibodies may be associated with the neoplastic development of these histotypes. Hence, the monoclonal antibodies of the present invention represent a new diagnostic indicator of certain cancers in these tissues. Because certain adenocarcinomas do not contain detectable levels of the epitope, the monoclonal antibodies identify antigenic differences which can be prognostically significant.

In addition to their use as in vitro immunohistological reagents for cancer diagnosis, the monoclonal antibodies of the present invention can be used diagnostically in vivo. Because of their ability to target breast carcinoma cells in vivo, the monoclonal antibodies can be used in tumor localization and in the monitoring of metastases.

Furthermore, the monoclonal antibodies provided herein can be used as in vitro immunoserological and immunocytological reagents on body fluids to detect the presence of the specific antigen and/or cells bearing antigen. The monoclonal antibodies thereby permit non-invasive diagnosis of certain breast carcinomas and other cancers.

Most importantly, the hybridoma-derived monoclonal antibodies of the present invention represent new immunotherapeutic agents for the treatment of human breast cancer. The monoclonal antibodies are capable of inducing a rapid and significant reduction of progressively growing adenocarcinomas associated with the human breast. The tumoricidal effectiveness at low doses of the monoclonal antibodies, the high incidence of antigen expression in biopsies of breast cancer patients and the high specific binding of the monoclonal antibodies to certain breast tumors, the absence of modulation of the antigen recognized and the ability of the monoclonal antibodies to fix human complement and eradicate cancer cells independent of other cytotoxic agents are indications of the usefulness of the monoclonal antibodies in human breast cancer therapy.

The present invention provides methodologies useful in research for the evaluation of parameters

associated with the use of monoclonal antibodies against human tumors in passive human immunotherapy. The monoclonal antibodies can be used as probes to investigate the roles of antigen density, tumor growth rates, tumor size, cellular heterogeneity and other variables in the susceptibility of tumors to immunotherapy.

The invention contemplates the use of the monoclonal antibodies provided herein in covalent combination with cytotoxic or chemotherapeutic molecules. For instance, the monoclonal antibodies can be conjugated to certain cytotoxic compounds, including, but not limited to, radioactive compounds, diphtheria toxin (chain A), ricin toxin (chain A), adriamycin, chlorambucil or daunorubicin, to enchance their tumoricidal effectiveness. The invention also contemplates the covalent combination of the monoclonal antibodies with carbohydrate-active reagents such as, but not limited to, glycosidases, to unmask the cell surface antigen recognized, thereby increasing antibody binding and tumoricidal effectiveness.

The present invention further contemplates the use of the monoclonal antibodies in immunoadsorption procedures to effectively separate breast cancer cells from marrow elements based upon antibody binding, and in complement-mediated cytolytic procedures to eliminate malignant cells while sparing bone marrow stem cells. Because such procedures require a tumor antigen, such as the one recognized by the monoclonal antibodies of this invention, which is not found on bone marrow stem cells or lymphoid cells, the monoclonal antibodies represent a reagent useful for eliminating disseminated breast cancer cells from autologous bone marrow.

Because the monoclonal antibodies are produced by hybridoma techniques, the present invention provides theoretically immortal cell lines capable of consistently producing high titers of single specific antibodies against a distinct breast carcinoma antigen. This is a distinct advantage over the traditional technique of raising antibodies in immunized animals where the resulting sera contain multiple antibodies of differenct specificities that vary in both type and titer with each animal, and, in individual animals, with each immunization.

4. DESCRIPTION OF THE INVENTION

4.1. THE ANTIGEN

In the embodiment of the invention described in Section 5.2., MCF-7 breast carcinoma cells were used as 'antigen'. However, as demonstrated by experiments described in Section 5.6.1., 5.8.1., and 5.8.3., the epitope recognized by the antibody of this invention is present on the surface of several cultured cell lines as well as cells present in certain extra-mammary tumors. Hence, these cells also represent potential 'antigen' or sources of antigen with which to immunize animals to obtain somatic cells for fusion.

For example, the BT-20, MDA-MB-157, and to a lesser extent, SK-BR-3 breast carcinoma cell lines can be used as immunogens. Non-breast carcinoma cell lines, including PANC-1 (pancreas carcinoma) and HT-29 (colon carcinoma) also represent potential candidates as immunogen in other embodiments of the invention. Furthermore, cells derived from intensely-staining benign or malignant breast tissues listed in TABLE VIII, can be used. Similarly, cells derived from positively-staining

extra-mammary tumors listed in TABLE VIII, for instance ovarian, colon, endometrial, renal, bronchogenic, etc., carcinomas or ovarian cystadenomas, can be used as immunogen.

Cells or fluids derived from pleural effusions of breast cancer patients (see Section 5.7.3.) and antigen isolated from cell lysates as described in Section 5.7.4. are also potential candidates with which to immunize animals.

4.2. SOMATIC CELLS

Somatic cells with the potential for producing antibody and, in particular B lymphocytes, are suitable for fusion with a B-cell myeloma line. Those antibody-producing cells that are in the dividing plasmablast stage fuse preferentially. Somatic cells may be derived from the lymph nodes, spleens and peripheral blood of primed animals and the lymphatic cells of choice depends to a large extent on their empirical usefulness in the particular fusion system. Once-primed or hyperimmunized animals can be used as a source of antibody-producing lymphocytes. Mouse lymphocytes give a higher percentage of stable fusions with the mouse myeloma lines described in Section 4.3. However, the use of rat, rabbit, and frog cells is also possible.

Alternatively, human somatic cells capable of producing antibody, specifically B lymphocytes, are suitable for fusion with myeloma cell lines. While B lymphocytes from biopsied spleens or lymph nodes of individuals may be used, the more easily accessible peripheral blood B lymphocytes are preferred. The lymphocytes may be derived from patients with diagnosed breast or other adenocarcinomas.

4.3. MYELOMA CELLS

Specialized myeloma cell lines have been developed from lymphocyte tumors for use in hybridoma-producing fusion procedures [G. Kohler and C. Milstein, Eur. J. Immunol. 6:511-519 (1976); M. Schulman et al., Nature 276:269-270 (1978)]. The cell lines have been developed for at least three reasons. The first is to facilitate the selection of fused hybridomas among unfused and similarly indefinitely self-propogating myeloma cells. Usually, this is accomplished by using myelomas with enzyme deficiencies that render them incapable of growing in certain selective media that support the growth of hybridomas. The second reason arises from the inherent ability of lymphocyte tumor cells to produce their own antibodies. The purpose of using monoclonal techniques is to obtain immortal fused hybrid cell lines that produce the desired single specific antibody genetically directed by the somatic cell component of the hybridoma. To eliminate the production of tumor cell antibodies by the hybridomas, myeloma cell lines incapable of producing light or heavy immunoglobulin chains or those deficient in antibody secretion mechanisms are used. A third reason for selection of these cell lines is for their suitability and efficiency for fusion.

Several myeloma cell lines may be used for the production of fused cell hybrids, including X63-Ag8, NSI-Ag4/1, MPC11-45.6TG1.7, X63-Ag8.653, Sp2/0-Ag14, FO, and S194/5XX0.BU.1, all derived from mice, 210.RCY3.Ag1.2.3 derived from rats and U-226AR, and $GM1500GTGAL_2$, derived from rats and U-226AR, and $GM1500GTGAL_2$, derived from humans. [G.J. Hammerling, U. Hammerling and J.F. Kearney (editors), Monoclonal antibodies and T-cell hybridomas in: J.L. Turk (editor)

Research Monographs in Immunology, Vol. 3, Elsevier/North Holland Biomedical Press, New York (1981)].

4.4. FUSION

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion as in the example in Section 5.2. (though the proportion may vary from about 20:1 to about 1:1), respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. It is often preferred that the same species of animal serve as the source of the somatic and myeloma cells used in the fusion procedure. Fusion methods have been described by Kohler and Milstein [Nature 256:495-497 (1975) and Eur. J. Immunol. 6:511-519 (1976)], and by Gefter et al. [Somatic Cell Genet. 3:231-236 (1977)]. The fusion-promoting agent used by those investigators were Sendai virus and polyethylene glycol (PEG), respectively. The fusion procedure of the example of the present invention is a modification of the method of Kohler and Milstein, supra.

4.5. ISOLATION OF CLONES AND ANTIBODY DETECTION

Fusion procedures usually produce viable hybrids at very low frequency, about 1×10^{-6} to 1×10^{-8}. Because of the low frequency of obtaining viable hybrids, it is essential to have a means to select fused cell hybrids from the remaining unfused cells, particulary the unfused myeloma cells. A means of detecting the desired antibody-producing hybridomas among the other resulting fused cell hybrids is also necessary.

Generally, the fused cells are cultured in selective media, for instance HAT medium containing hypoxanthine, aminopterin and thymidine. HAT medium permits the proliferation of hybrid cells and prevents growth of unfused myeloma cells which normally would continue to divide indefinitely. Aminopterin blocks de novo purine and pyrimidine synthesis by inhibiting the production of tetrahydrofolate. The addition of thymidine bypasses the block in pyrimidine synthesis, while hypoxanthine is included in the media so that inhibited cells can synthesize purine using the nucleotide salvage pathway. The myeloma cells employed are mutants lacking hypoxanthine phosphoribosyl transferase (HPRT) and thus cannot utilize the salvage pathway. In the surviving hybrid, the B lymphocyte supplies genetic information for production of this enzyme. Since B lymphocytes themselves have a limited life span in culture (approximately two weeks), the only cells which can proliferate in HAT madia are hybrids formed from myeloma and spleen cells.

To facilitate screening of antibody secreted by the hybrids and to prevent individual hybrids from overgrowing others, the mixture of fused myeloma and B lymphocytes is diluted in HAT medium and cultured in multiple wells of microtiter plates. In two to three weeks, when hybrid clones become visible microscopically, the supernatant fluid of the individual wells containing hybrid clones is assayed for specific antibody. The assay must be sensitive, simple and rapid. Assay techniques include radioimmunoassays, enzyme immunoassays, cytotoxicity assay, and plaque assays.

4.6. CELL PROPAGATION AND ANTIBODY PRODUCTION

Once the desired fused cell hybrids have been selected and cloned into individual antibody-producing

cell lines, each cell line may be propagated in either of two standard ways. A sample of the hybridoma can be injected into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can be tapped to provide monoclonal antibodies in high concentration. Alternatively, the individual cell lines may be propogated in vitro in laboratory culture vessels; the culture medium, also containing high concentrations of a single specific monoclonal antibody, can be harvested by decantation, filtration or centrifugation.

4.7. IN VITRO DIAGNOSTIC USES FOR MONOCLONAL ANTIBODIES TO HUMAN BREAST CARCINOMA

4.7.1. IMMUNOHISTOLOGICAL AND IMMUNOCYTOLOGICAL APPLICATIONS

The monoclonal antibodies of this invention can be used as probes in detecting discrete antigens in human tumors. The expression or lack of expression of these antigens can provide clinically exploitable information which is not apparent after standard histopathological evaluations. It may thus be possible to correlate the immuno-phenotypes of individual tumors with various aspects of tumor behavior and responsiveness to certain types of therapies, thus establishing important classifications of prognosis.

Monoclonal antibodies produced by the hybridoma methodologies herein described can be used to detect potential breast carcinoma cells in histological and

cytological specimens and in particular, to distinguish well-differentiated from poorly-differentiated tumors based on staining patterns and intensities. For instance, using the immunoperoxidase staining technique described in Section 5.8.1., it has been observed that the monoclonal antibodies of its invention stained an apical membrane-associated moiety in sections of benign lesions and well-differentiated adenocarcinomas of the breast; less-differentiated tumors however, generally exhibited a cytoplasmic staining pattern. While normal breast tissue also exhibited the surface staining pattern of benign and well-differentiated lesions, the intensity of staining was considerably less (See TABLE VIII, Section 5.8.3.).

Another important in vitro diagnostic application of the monoclonal antibodies of this invention is the evaluation of adenocarcinomas other than breast. The antibody has been used to detect the epitope in adenocarcinomas of several histotypes including colon, ovary, uterus, pancreas and prostate, while the normal counterparts at these histological sites did not express the determinant. Thus, the novel monoclonal antibody detects an antigenic determinant in these adenocarcinomas which is tumor-associated. For example, no normal ovaries have displayed detectable levels of the epitope, whereas 15/15 ovarian carcinomas have expressed the determinant. Since the expression of this antigenic determinant appears to be associated with neoplastic development in the ovary, the monoclonal antibody may be a useful immunodiagnostic reagent for ovarian carcinomas. Other adenocarcinomas (gastric, jejunal, colonic, pancreatic, prostatic and bronchogenic) exhibited varying incidences of antigen expression. Since every adenocarcinoma specimen did not contain detectable levels of the determinant, the monoclonal antibody identifies antigenic differences which may be prognostically significant.

As an alternative to immunoperoxidase staining, immunofluorescent techniques can be used to examine human specimens with monoclonal antibodies to breast carcinoma. In a typical protocol, slides containing cryostat sections of frozen, unfixed tissue biopsy samples or cytological smears are air dried and incubated with the monoclonal antibody preparation in a humidified chamber at room temperature.

The slides are then layered with a preparation of antibody directed against the monoclonal antibody, usually some type of antimouse immunoglobulin if the monoclonal antibodies used are derived from the fusion of a mouse spleen lymphocyte and a mouse myeloma cell line. This antimouse immunoglobulin is tagged with a compound that fluoresces at a particular wavelength for instance rhodamine or fluorescein isothiocyanate. The staining pattern and intensities within the sample are then determined by flourescent light microscopy and optionally photographically recorded.

4.7.2. IMMUNOSEROLOGICAL APPLICATIONS

The use of the monoclonal antibodies described herein can be extended to the screening of human biological fluids for the presence of the specific antigenic determinant recognized. In vitro immunoserological evaluation of sera withdrawn from patients thereby permits non-invasive diagnosis of cancers. By way of illustration, human fluids, such as pleural fluids or lymph, can be taken from a patient and assayed for the specific epitope, either as released antigen or membrane-bound on cells in the sample fluid, using the anti-breast carcinoma monoclonal antibodies in standard radioimmunoassays or enzyme-linked immunoassays known in the art.

4.8. IN VIVO DIAGNOSTIC AND THERAPEUTIC USES FOR MONOCLONAL ANTIBODIES TO HUMAN BREAST CARCINOMA

4.8.1. TUMOR LOCALIZATION

The monoclonal antibodies of this invention are capable of targeting breast carcinoma cells in vivo. They can therefore be used in humans for tumor localization and for monitoring of metastases. For this application, it is preferable to use purified monoclonal antibodies. Purification of monoclonal antibodies for human administration by ammonium sulfate or sodium sulfate precipitation followed by dialysis against saline and filtration sterilization has been described by Miller et al. [in: Hybridomas in Cancer Diagnosis and Therapy, (1982) supra, p. 134,] and by Dillman et al. [Id. p.155] which are hereby incorporated by reference. Alternatively, immunoaffinity chromatography techniques may be used to purify the monoclonal antibodies.

The purified monoclonal antibodies can be labelled with radioactive compounds, for instance, radioactive iodine, and administered to a patient intravenously. After localization of the antibodies at the tumor or metastatic site, they can be detected by emission tomographical and radionuclear scanning techniques thereby pinpointing the location of the cancer. Experimental radioimmunodetection with monoclonal antibodies by external scintigraphy has been reported by Solter et al. [Id., p. 241] hereby incorporated by reference.

4.8.2. PASSIVE IMMUNOTHERAPY FOR TREATMENT OF HUMAN CANCER

Because the monoclonal antibodies of this invention are capable of inducing rapid and significant volume reduction of established, progressively growing solid tumors associated with the human breast, they may be used in the treatment of human breast cancers, both in females and males, and possibly in the treatment of other adenocarcinomas, particularly ovarian cancer. By way of illustration, purified anti-breast carcinoma monoclonal antibody (see Section 4.8.1) is suspended in an appropriate carrier, e.g., saline, with or without human albumin, at an appropriate dosage and is administered to a patient. The monoclonal antibodies are preferably administered intravenously, e. g., by continuous intravenous infusion over several hours, as in Miller et al., incorporated by reference, supra. Infusions can be administered over a period weeks during which the antitumor effects are monitored.

The monoclonal antibodies described herein may be used clinically in conjunction with bone marrow transplantation or replacement therapies. Bone marrow tapped from patients undergoing treatment can be contacted with the anti-breast carcinoma monoclonal antibodies to eliminate contaminating cancer cells bearing the specific epitope by immunoadsorption. Cells may also be eliminated by complement mediated cytolytic procedures. Because the antigenic determinant recognized by the anti-breast carcinoma monoclonal antibody is absent on bone marrow stem cells and lymphoid cells, the monoclonal antibodies can effectively remove breast carcinoma cells from autologous or allogenic bone marrow prior to transplantation or reinfusion.

4.8.3. TREATMENT OF HUMAN CANCER WITH MONOCLONAL ANTIBODY CONJUGATES

The monoclonal antibodies of this invention can be used in conjunction with a broad spectrum of pharmaceutical or cytotoxic agents such as: radioactive compounds (e.g., I^{125}, I^{131}); agents which bind DNA, for instance, alkylating agents or various antibiotics (e.g., daunomycin, adriamycin, chlorambucil); antimetabolites such as methotrexate; agents which act on cell surfaces (e.g., venom phospholipases and microbial toxins); and protein synthesis inhibitors (e.g., diphtheria toxin and toxic plant proteins). [For reviews on the subject, see Bale et al., Cancer Research 40:2965-2972 (1980); Ghose and Blair, J. Natl. Cancer Inst. 61(3):657-676 (1978) Gregoriadis, Nature 265:407-411(1988); Gregoriadis, Pharmac. Ther. 10:103-108 (1980); Trouet et al., Recent Results Cancer Res. 75:229-235 (1980)]. Of particular importance are those agents capable of exerting toxic effects at the level of the cell surface, such as adriamycin [Tritton, T.R. and Yee. G., Science, 217:248-50 (1982)].

The methods used for binding the cytotoxic agents to the monoclonal antibody molecule can involve either non-covalent or covalent linkages. Since non-covalent bonds are more likely to be broken before the antibody complex reaches the target site, covalent linkages are preferred. For instance, carbodiimide can be used to link carboxy groups of the pharmaceutical agent to amino groups of the antibody molecule. Bifunctional agents such as dialdehydes or imidoesters can be used to link the amino group of a drug to amino groups of the antibody molecule. The Schiff base reaction can be used to link drugs to antibody molecules. This method involves the periodate

oxidation of a drug or cytotoxic agent that contains a glycol or hydroxy group, thus forming an aldehyde which is then reacted with the antibody molecule. Attachment occurs via formation of a Schiff base with amino groups of the antibody molecule. Additionally, drugs with reactive sulfhydryl groups have been coupled to antibody molecules.

Similarly, glycosidic enzymes such as neuraminidase or α-mannosidase can be conjugated to the monoclonal antibodies.

Conjugated antibodies can be administered to patients, as in Section 4.8.2., to achieve enhanced tumoricidal effects through the cytotoxic action of the chemotherapeutic agents or the increased binding effect of the glycosidase (See Section 5.7.5).

5. EXAMPLES

5.1. BREAST CELL LINES AND TISSUES

Breast carcinoma cell lines used included the following: MCF-7, derived from pleural effusion of scirrhous carcinoma [Soule, D.H. et al., J. Natl. Cancer Inst., 51:1409-1416(1973)]; SK-BR-3, derived from pleural effusion of adenocarcinoma [Fogh, J. and Trempe, G., New Human Tumor Cell Lines In: J. Fogh (ed.) Human Tumor Cells In Vitro, pp. 115-153, New York: Plenum Press (1975)]; BT-20, derived from primary carcinoma [Lasfargues, E.Y. and Ozzello, L., J. Natl. Cancer Inst. 21:1131-1147 (1958)] and MDA-MB-157, derived from pleural effusion of medullary carcinoma [Young, R.K. et al., In Vitro, 9:239-245 (1974)]. Each of the carcinomas is tumorigenic in nude mice. Cultures of apparently normal mammary cells used included: HBL-100, epithelial cells derived from a

human milk sample [Polanowski, F.P. et al., In Vitro 12:328-336 (1976)] and HS0578 Bst, myoepithelial cells derived from normal tissue adjacent to primary carcinoma [Hackett, A.J. et al., J. Natl. Cancer Inst., 58:1795-1806 (1977)]. Neither normal breast cell line is capable of producing tumors in nude mice. Murine mammary tumor cell line MMT 060562 was obtained from the American Type Culture Collection.

Tissue specimens representing normal, benign, and malignant breast lesions, along with other tissues, were obtained from the Pathology Department of St. Joseph's Hospital, Buffalo, N.Y. All specimens were histopathologically assessed.

5.2. IMMUNIZATION AND CELL FUSION

Balb/c mice received, on days 0, 7 and 14, intraperitoneal injections ($5x10^6$ cells per 0.2 ml) of live breast cancer cells which were suspended in Dulbecco's phosphate-buffered saline (D-PBS). On day 60, mice received an intravenous challenge of $2x10^6$ cells in D-PBS. Cell fusion was carried out 3 days later according to the procedure developed by Kohler and Milstein [Nature (Lond.) 256:495-497 (1975)], with modifications: Balb/c mouse splenocytes ($1x10^8$ cells) were fused in 0.15M HEPES (hydroxy-ethylpiperazine-ethanesulfonic acid) buffer (pH 7.5) comprising 40% (v/v) polyethylene glycol (PEG, MW=3,400) and 10% (v/v) dimethyl sulfoxide (DMSO) with $5x10^7$ mouse myeloma cells (P3x63Ag8.653). Fused cells were distributed to 96-well culture dishes and cultured in selective hypoxanthine-aminopterin-thymidine (HAT) medium at 37°C with 7.5% CO_2 in a humid atmosphere. Two to three weeks later, supernatants were assayed for direct binding activity on breast cancer cell lines. Hybridomas

were detected in over 3,000 culture wells, with approximately 5% secreting antibodi s reactive against MCF-7 breast cancer cells as determined by cell-surface radioimmunoassay (see Section 5.5.). Cultures showing specificity towards breast cancer were cloned by limiting dilution and subcloned in agarose [See e.g., Schreier, M. et al., Hybridoma Techniques, pp. 11-15, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1980)]. Stable cultures of antibody-producing hybridomas were expanded in complete media [RPMI-1640 media supplemented with 10% (w/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 µg/ml insulin (GIBCO Grand Island, NY)] and exhausted culture fluids were the only source of antibodies used.

Following this procedure, hybridoma cell line F36/22, derived from immunization with MCF-7 cells, was obtained. The hybridoma was grown in mass culture in complete medium to produce monoclonal antibody for characterization studies and other applications described below.

5.3. ISOTYPING OF F36/22 MONOCLONAL ANTIBODIES

To determine the class of immunoglobulin produced by the F36/22 hybrid, 10^7 F36/22 hybridoma cells were washed in D-PBS, collected by centrifugation and lysed in 100 µl of 0.5% Nonidet P-40 for 20 minutes on ice. Ten µl of the 40,000 xg supernatant were examined by immunodiffusion against antisera specific for each of murine immunoglobulin classes: IgM, IgG1, IgG2a, IgG2b and IgG3 (Miles Laboratories, Elkhart, Ind). Isotype analysis revealed that monoclonal antibody produced by F36/22 (hereinafter McAb F36/22 or monoclonal antibody F36/22) was an immunoglobulin of the γ3 class.

5.4. IMMUNOGLOBULIN PREPARATIONS

The monoclonal antibody F36/22 and control murine γ3 immunoglobulin were isolated in an identical manner using Staphylococcal aureus protein A-Sepharose (Pharmacia, Piscataway, N.J.) as desribed [Ey, P. L. et al., Immunochem., 15: 429-36 (1978)]. Diluted serum or ascites or clarified culture fluid were applied to the protein A-Sepharose adsorbent followed by removal of non-binding components with pH 6.0 buffer. Immunoglobulin of the γ3 subclass was eluted at pH 4.5, neutralized with sodium hydroxide (NaOH) and dialyzed into PBS (phosphate buffered saline; 10 mM phosphate, pH 7.4). All immunoglobulin preparations were adjusted to 1 mg/ml and frozen in small aliquots until needed.

Immunoglobulin preparations used for the present studies were at least 95% pure as judged by polyacrylamide gel electrophoresis [Laemmli, U.K., Nature 227: 680-85 (1970)].

5.5. CELL-SURFACE RADIOIMMUNOASSAY (CS-RIA)

Unless otherwise indicated, target cells were fixed in 2% p-formaldehyde/D-PBS for 10 minutes at room temperature and stored in the presence of assay buffer [D-PBS/0.1% gelatin/1 mM PMSF (phenyl methyl sulfonyl fluoride)/0.05% azide] at 4°C for up to 2 weeks. To perform the assay, $1x10^5$ cells were incubated with 100 µl of culture fluid from a culture of the F36/22 hybridoma for 2 hours at room temperature. Cells were washed 3 times with assay buffer before the addition of rabbit anti-mouse immunoglobulin diluted 1/500 in assay buffer [Brown, J. P. et al., J. Immunol. Methods, 31: 201-209 (1979)]. After 1 hour at room temperature, cells were

washed and treated with ^{125}I-labelled Staphylococcal Protein-A (Pharmacia, Piscataway, N.J.) at 2.5×10^5 cpm per dose. After 1/2 hour, the cells were washed, dissolved in 2N NaOH and transferred to tubes, where bound radioactivity was measured.

5.6. IN VITRO CHARACTERIZATION OF MONOCLONAL ANTIBODY F36/22

5.6.1. DETECTION AND BINDING OF McAb F36/22 TO CULTURED CELLS

McAb F36/22 was tested by CS-RIA against a panel of cell types (TABLE I). McAb F36/22 produced a strong reaction (greater than 10,000 cpm) against breast cancer cells MCF-7 and BT-20 and a significant reaction versus the other tested breast cancer lines. This antibody weakly bound to a colon carcinoma and to a pancreas carcinoma (10 to 20% of maximum binding relative to MCF-7 cells). Other cells tested, including lymphoid/leukemoid cells, fibroblasts, and erythrocytes and cultures representing apparently normal mammary epithelial cells [Polanowski, F. P. et al. (1976), supra] and myoepithelial cells [Hackett, A. J. et al. (1977), supra] also failed to bind significant levels of antibody F36/22 (less than 10% of maximum binding). The results obtained from the cell surface binding assay, as summarized in TABLE I, indicate that the specificity recognized by McAb F36/22 is expressed maximally on selected breast cancer cells.

TABLE I

CELL-SURFACE BINDING OF ANTIBODY F36/22[a]

Target Cell	% of maximum binding activity[b] F36/22
Breast Carcinoma	
BT-20	99
SK-BR-3	23
MCF-7	100
MDA-MB-157	44
Mouse Mammary Tumor MMT 060562	5
Pancreas Carcinoma	
BxPC-3	8
PANC-1	13
Prostate Carcinoma	
LNCaP	3
PC-3	6
Colon Carcinoma	
HT-29	16
Lung Carcinoma	
PC3	4
CHAGO	5
Rhabdomyosarcoma	
A-204	2
Melanoma	
Palarmo	3
Thyroid Carcinoma	
TT-4	1
Lymphoblastoid/Leukemoid	
RAJI (B-Cell)	6
DAUDI (B-Cell)	8
BALM-3 (B-Cell)	5
RPMI-6410 (B-Cell)	9
PEER (T-Cell)	5
MOLT-4 (T-Cell)	4
U-937 (monocytoid)	9
K-562 (erythroleukemoid)	10

Target Cell	% of maximum binding activity[b] F36/22
Normal Breast	
HBL-100 (epithelial)	2
HSO578 Bst (myoepithelial)	2
Erythrocytes	
Type A	3
Type B	5
Type O	4
Type O (neuraminidase-digested)[c]	3
Fibroblasts	
BG-9	8
Peripheral Blood Lymphocytes	3

a. Antibody binding activity was assessed by the cell-surface radioimmunoassay (CS-RIA).

b. Maximum binding (100%) represents the cell line binding the most antibody, and binding to other cells was expressed relative to that level. The maximum cpm (mean of 2 experiments) was as follows: antibody F36/22; 10,743 cpm.

c. Neuraminidase Digestion: Washed, type "O" erythrocytes were treated with C. perfringens neuraminidase (Boehringer-Mannheim, Indianapolis, In.) at 1 U enzyme/ml of 0.1M acetate/0.15M NaCl, pH 5.5. After 1 hour at 37°C, the cells were washed with CS-RIA assay buffer and assayed for antibody binding activity as compared to acetate buffer-treated control erythrocytes.

5.6.2. QUANTITATIVE ADSORPTION OF McAb F36/22 TO CULTURED CELLS

Adsorption tests were performed to determine if low binding levels obtained from the direct-binding assays presented in Section 5.6.1. were reflecting low levels of antigen, rather than absence of antigen. As shown

previously [Dippold, W. G. et al., Proc. Natl. Acad. Sci. U.S.A., 77: 6114-6118 (1980)], target cells tested against certain monoclonal antibodies may be direct-binding-test-negative/adsorption- test-positive. The number of cells required to adsorb 50% of antibody activity was calculated and presented as an AD_{50} value according to the following procedure.

Suspensions containing different numbers of human cells (10^3 to greater than 10^7 cells/ml) were added to equal volumes (total volume=200 µl) of hybridoma F36/22 culture supernatant fluid (approximately 38 ng murine IgG) and incubated for 1 hour on ice. The human cells used were: breast carcinoma BT-20, MCF-7, and MDA-MB-157; pancreas carcinoma PANC-1; colon carcinoma HT-29; lymphoblastoid RAJI; normal breast (epithelial) HBL-100; fibroblast BG-9; and red blood cells. After centrifugation, the binding activity remaining in the supernatant was measured using the CS-RIA.

Appropriate dilutions of hybridoma culture supernatant fluid were established by titration analysis of each antibody against breast cancer target cells. A final dilution of antibody was chosen which was midway down the slope, representing 50% of maximum binding activity. This corresponded to 38 ng antibody/ml final dilution for F36/22.

From the cpm of [^{125}I] Protein A bound to target cells, the estimated number of adsorbing cells required to remove 50% of the antibody activity was interpolated from a regression line of best fit.

The results of adsorption analyses indicated that the capacity to adsorb McAb F36/22 of the various cell

types tested was greatest in selected breast cancer cells. Cultures of breast carcinoma cells BT-20, MCF-7 and MDA-MB-157 were able to adsorb F36/22 activity at AD_{50} values of $3x10^5$, $5x10^5$, and $2x10^7$, respectively. Fibroblasts and erythrocytes, along with lymphoblastoid, and colon carcinoma cells, showed no ability to adsorb McAb F36/22. Pancreas carcinoma cells demonstrated an AD_{50} value of $1.6x10^8$; this indicated very low levels of epitope F36/22, approximately 500-fold less than on BT-20 breast carcinoma.

5.6.3. RECIPROCAL BINDING INHIBITION

Reciprocal binding inhibition experiments were performed to determine whether McAb F36/22 recognized the same, closely associated or different antigenic determinants as antibodies to: fibronectin, human DR-antigen, α-fetoprotein, pregnancy-associated α-2-macroglobulin, carcinoembryonic antigen (CEA), class I HLA-A, B or C monomorphic determinants, ß-2-microglobulin or ferritin.

The procedure used to test reciprocal binding inhibition was as follows:

F36/22 hybridoma antibodies were internally labelled by the addition of 5 µCi of [^{14}C] leucine (New England Nuclear, Boston, Ma.) to cultures of $2x10^6$ cells in 3 ml of leucine-free Modified Eagle's Medium (MEM) supplemented with 5% dialyzed fetal bovine serum. After 18 hours under standard culture conditions, culture fluids were clarified by centrifugation (20,000 xg for 1/2 hour) and dialyzed against D-PBS/1 mM leucine/1 mM PMSF/0.05% azide. For competitive analysis, 50 µl of unlabelled antibodies (50-fold diluted sera or ascites) or 50 µl

CS-RIA assay buffer (control) were allowed to react with target cells for 1 hour prior to the addition of ^{14}C-labelled antibodies (50 µl). After 3 hours the cells were washed 4 times with CS-RIA assay buffer and counted. The 100% binding level was 2,200 cpm. Antibodies used for these studies included: anti-HLA monoclonal antibody W6/32 (Accurate Chemical Corp., Hicksville, N.Y.); anti-DR monoclonal antibody MAS-019 (Accurate Chemical Corp.); anti-CEA polyclonal antiserum [Chu, T. M. and Nemoto, T., J. Natl. Cancer Inst. 51:1119-1122 (1973)]; anti-α-fetoprotein polyclonal antiserum (obtained from H. Hirai, Hokkaido University, Japan); anti-pregnancy-associated macroglobulin (obtained from E. J. Sarcione, Roswell Park Memorial Institute); anti-fibronectin monoclonal antibody MAS-037 (Accurate Chemical Corp.); anti-ß- 2-microglobulin polyclonal antiserum (Accurate Chemical Corp.) and antiserum to human ferritin (obtained from E. J. Sarcione, Roswell Park Memorial Institute).

The per cent inhibition of McAb F36/22 binding activity by anti-CEA antibodies, anti-α-fetoprotein antibodies, and anti-ß-2-microglobulin antibodies was approximately 5% or less. The per cent inhibition by anti-fibronectin antibodies, anti-HLA antibodies, anti-DR-antigen antibodies, anti- pregnancy-associated macroglobulin antibodies, and anti-human ferritin antibodies was approximately 12% or less.

5.6.4. IN VITRO CYTOTOXICITY OF McAb F36/22

Complement-mediated cytotoxicity of monoclonal antibody was measured using the procedure of Brown, et al. [J. Immunol. Methods, 30: 23-35 (1979)]. Cells were incubated in the presence of McAb F36/22 plus rabbit

complement for 1.5 hours after which time cells were evaluated for their ability to metabolically incorporate radioactive leucine as compared to untreated controls. Cells examined included Chago lung carcinoma [Rabson, A. S. et al., J. Natl. Cancer Inst., 50: 660-74 (1973)], Daudi lymphoma [Klein, E. et al., Cancer Res., 28: 1300-10 (1968)] and MCF-7 breast carcinoma [Soule, H. D. et al., J. Natl. Cancer Inst., 51: 1409-16 (1973)].

Titration data summarized in TABLE II demonstrate that McAb F36/22 is cytotoxic to MCF-7 breast carcinoma cells in the presence of complement. This cytotoxic effect has been demonstrated at high dilutions of antibody. Also, the cytotoxicity observed is specific for antigen-expressing tumor cells. Cell lines expressing undetectable amounts of determinant, such as Chago lung carcinoma and Daudi lymphoma, were not significantly killed in the presence of complement plus McAb F36/22 at high dilutions which were cytotoxic to breast cancinoma.

TABLE II

IN VITRO CYTOTOXICITY OF McAb F36/22

Approx. % Cytotoxicity at Various Antibody Dilutions

Cell Line	Dilutions					
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
MCF-7	65	60	65	25	10	0
Chago	55	0	0	0	0	0
Daudi	60	0	0	0	0	0

In vitro antibody mediated complement cytotoxicity assays were repeated on BT-20 and MCF-7 cell lines. The results showed specific cell lysis (greater than 50% cell kill) at a concentration of 1 ng of McAb F36/22 per ml of medium and no cytolysis of 11 different non-breast cell lines (8 leukemia, 2 carcinomas, 1 lymphoma) at a concentration of 2.5 µg/ml. Rabbit or human complement at 1:10 dilution were both shown to be equally effective. Antibody without complement gave no significant cytolysis.

The rapidity of the tumoricidal process (see Section 5.9.3.) and the in vitro indication of specific cytolysis at very low concentration of McAb F36/22 with complement point to the involvement of a complement mediated effect. It has been reported that murine $\gamma 3$ antibodies bind with low affinity and low frequency to murine macrophages [Ralph, P. et al., J. Immunol, 125:1885-1888 (1980)]. The possibility exists, however, that antibody mediated cellular cytotoxicity also takes place.

5.6.5. REACTIVITY OF McAb F36/22 WITH ESTROPHILIN COMPLEXES

Experiments were performed to define the possible role of estrophilin in the reaction of McAb F36/22 against human breast carcinomas. Using an immunochemical assay, McAb F36/22 added prior to labelled estradiol was unable to competitively inhibit the formation of estradiol-estrophilin complexes. However, the possibility existed that even through F36/22 did not react with an epitope involved in the hormone-receptor binding site, it may detect an epitope located elsewhere on the estrophilin molecule. To evaluate this, the following procedure was used.

Radiolabelled estradiol-estrophilin complexes were prepared using MCF-7 cytosol as described by Horwitz and McGuire [Cancer Res., 37: 1733-1738 (1977)], except that ^{125}I-labelled estradiol was used (New England Nuclear, 2000 µCi/mM) in place of the tritiated reagent. Preformed complexes were incubated at 4°C for 1 hour with McAb F36/22 (25 µg) or control immunoglobulin (25 µg) from serum of a non-immunized mouse as described previously [Greene, G. L. et al., Proc. Natl. Acad. Sci. U.S.A., 77: 5115-5119 (1980)]. Each mixture was individually applied to a column (1x45 cm) packed with S-200 chromatography media and equilibrated with running buffer (10 mM Tris-HCl, pH 7.4 containing 1.5 mM DTT (dithiotreitol), 1 mM EDTA (ethylenediaminetetraacetic acid) and 400 mM KCl). Successive 1 ml fractions were collected and radioactivity was measured. Purified human hemoglobin (Sigma) was used as an internal marker of the 4S protein peak.

The estradiol-estrophilin complexes eluted in the 4S region both in the presence and absence of McAb F36/22 as compared to monoclonal antibodies which react with estrophilin [Greene, G. L. et al. (1980), supra], thus indicating no reactivity with the estrophilin molecule.

5.6.6. RELATIONSHIP BETWEEN ESTRADIOL CONCENTRATION AND McAb F36/22 BINDING LEVELS

Hormonal involvement of antigen expression in MCF-7 breast cancer was investigated. A study was performed to ascertain if increased antigen expression of estrogen receptor-rich (ER-rich) tumors was related to the estradiol concentration.

First, endogenous estradiol was removed from fetal bovine serum (FBS) used in normal complete cell culture medium. The basic procedure used was reported earlier by Westley and Rochefort [Cell, 20: 353-362 (1980)]. Fifty ml of FBS # 309 (GIBCO) was depleted of estrogen as follows: A DCC suspension (0.025% Norit A and 0.0025% dextran in TESH (10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 1.5 mM DDT buffer) was prepared according to the method of Garola and McGuire [Cancer Res., 38: 2216-2220 (1978)]. The DCC (250 mg charcoal, 25 mg of dextran per 50 ml of FBS) was added to the FBS and mixed on an aliquot shaker at 55°C for 30 minutes. After 2 treatments with DCC, the steroid-depleted FBS was sterile filtered through 0.2 µm Nalgene filters. Complete medium consisted of RPMI-1640 containing 10% estrogen-depleted FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. All tissue culture reagents were obtained from GIBCO, Grand Island, N.Y.

The following protocol was used to determine the effect of exogenous estradiol on antibody binding levels. MCF-7 cells were washed twice in D-PBS, resuspended in steroid-depleted medium, and lightly seeded into replicate flasks. The medium was replaced every third day. On day 10, various final concentrations of estradiol-17 ß-cypionate (Sigma) (0, 10^{-3}, 10^{-6}, 10^{-9}M) in DCC-treated medium were added to the flasks. After 72 hours of stimulation, the cells were harvested by gentle aspiration, counted using a hemocytometer and aliquoted. Cells were washed twice in D-PBS and then fixed for 10 minutes in 2% p-formaldehyde in 30 mM phosphate buffer, pH 7.2. The cells were again washed and treated with 0.5% saponin in 30 mM phosphate buffer, pH 7.2, for 3 minutes to reveal intracellular binding sites [Fambrough, D. M. and Devreotes, P. N., J. Cell. Biol., 76: 237-244

(1978)]. The cells were further washed and incubated with [^{125}I]-F(ab')$_2$ fragments of McAb F36/22 for 60 minutes (200,000 cpm/tube), washed 4 times, and the counts were measured in a Packard gamma spectrometer.

Competitive inhibition, using saturating doses (10 µg/ sample) of unlabelled McAb F36/22 prior to the addition of the labeled F(ab')$_2$ fragments permitted an estimation of non-specific background. In addition, an irrelevant IgG3 control antibody (10 µg/sample) was incubated prior to the labelled fragments of McAb F36/22, in order to assess the specificity of the binding reaction.

Using these in vitro conditions, exogenous estradiol concentrations of 10^{-3} and 10^{-6}M were shown to significantly increase the amount of antibody binding levels as compared to control-treated cultures (p less than .001) (TABLE III). Fixation of the MCF-7 cells with p-formaldehyde alone failed to elicit any significant changes in cell-surface binding in response to increasing concentrations of estradiol. However, treatment of the cells with saponin, which reveals intra-cytoplasmic binding sites, greatly promoted the binding of McAb F36/22. This in vitro observation coincides with the in vivo tumor staining patterns (see Section 5.9.), where the predominant location of immunoperoxidase staining in ER-rich tumors is cytoplasmic (80%).

TABLE III

RELATIONSHIP BETWEEN ESTRADIOL LEVELS AND ANTIGEN EXPRESSION OF MCF-7 CELLS[a]

Final Estradiol Concentration	[^{125}I]-F(ab')$_2$ cpm bound/ 10^5 MCF-7 Cells[b]
0	3975 ± 556
10^{-9}M	4213 ± 842[c]
10^{-6}M	5333 ± 640[d]
10^{-3}M	6133 ± 306[d]

a. MCF-7 cells, maintained in estrogen-depleted medium for 10 days, were stimulated with various final concentrations of estradiol. After 72 hours, the cells were harvested, counted and aliquoted into 3 tubes/flask. The cells were fixed in buffered 2% p-formaldehyde for 10 minutes, and further treated with 0.5% buffered-saponin for 3 minutes to reveal intracellular binding sites. The cells were then incubated with [^{125}I]-F(ab')$_2$ fragments of McAb F36/22 for 60 minutes (200,000 cpm/tube), and the counts bound were measured in a gamma spectrometer.

b. In this experiment, specific binding of McAb F36/22 fragments was calculated by subtracting the non-specific binding in the presence of saturating levels of unlabelled McAb F36/22 from the total counts bound. Results were expressed as [^{125}I]-F(ab')$_2$ cpm bound/10^5 MCF-7 cells.

c. Not statistically significant.

d. Statistically significant antibody binding (p less than 0.05) as compared to control treated cultures (Student's t test).

Because exogenous estradiol was capable of increasing the cytoplasmic binding of McAb F36/22 to saponin-treated MCF cells, the antigen which McAb F36/22 recognizes may represent a protein whose sythesis is regulated under the estrogen-response machinery of the breast cancer cell. The existence of several estrogen-regulated proteins has been well established [see e.g., Butler, W. B. et al., Biochem. Biophys. Res. Commun., 90: 1328-1334 (1979); and Capany, F. et al., Biochem. Biophys. Res. Commun., 108: 8-15 (1982)] and monoclonal antibodies to estrogen-receptor status-related components have been described [Ciocca, D. R. et al., Cancer Res., 42: 4256-4258 (1982)]. Identification of such proteins may add new information regarding hormonally-dependent regulatory controls involved in breast cancer which may ultimately lead to new therapies.

5.7. CHARACTERIZATION OF THE ANTIGEN RECOGNIZED BY MONOCLONAL ANTIBODY F36/22

5.7.1. SOLID-PHASE ADSORPTION OF BT-20 BREAST CARCINOMA ANTIGEN BY LECTINS

The following solid-phase ligands were tested for their capacity to react with antigen occurring in BT-20 breast cancer cell lysates: Concanavalin A-Sepharose CL-4B (Pharmacia), in 50 mM sodium acetate/1M NaCl/1mM $CaCl_2$/1mM $MnCl_2$, pH 6.0 (binding capacity 8.5 mg/ml gel); Wheat-germ- Sepharose 6MB (Pharmacia), in D-PBS (binding capacity 5 mg/ml; Peanut Agglutinin-Ultrogel (LKB, Rockville, Md.), in D-PBS (binding capacity 4 mg/ml); and Gelatin-Agarose (Bio-Rad, Richmond, Ca.), in D-PBS (binding capacity 6 mg/ml).

Samples of BT-20 breast cancer cell lysates (8 mg protein/ml) were prepared in D-PBS/0.5% sodium

deoxycholate and were incubated with an equal volume of solid-phase ligand (25% v/v suspension in the appropriate buffer). After 1 hr at 4°C, the beads were washed 3 times and incubated with F36/22 hybridoma culture fluid for 2 hours at 4°C. Subsequently, antibody binding activity was revealed using the CS-RIA (Section 5.5.) procedure as for target cells. Since standard preparations of purified BT-20 breast cancer antigen was not available in this set of experiments as reference material, results of these assays were used to obtain qualitative information regarding lectin binding activities.

Concanavalin A was able to bind soluble components from BT-20 breast cancer cell lysates which in turn exhibited the capacity to adsorb McAb F36/22. Peanut agglutinin and wheat germ lectins had a more limited ability to bind F36/22 antibody-reactive components from BT-20 cell lysates (less than 20% of that exhibited by concanavalin). Also tested was insolubilized gelatin, which produced no antibody binding activity after treatment with breast cancer cell lysates.

5.7.2. ANTIGEN MODULATION

The stability of cell surface antigen after treatment of MCF-7 cells with McAb F36/22 was investigated.

To sensitize viable MCF-7 breast carcinoma cells growing in microwell plates, the cells were allowed to incubate in the presence of saturating doses of monoclonal antibody (10 µg/ml of sterile complete medium). The experiment was run at 0°, 23° and 37°C. At successive time intervals up to 4 hours the cells were washed and assayed for McAb F36/22 binding as described in Section 5.5. Additionally, monoclonal antibody- sensitized cells

were also examined under immunofluorescence using fluorescent labelled antibody (where the fluorescent label was fluorescein isothiocyanate (FITC)) against murine immunoglobulin (Accurate Chemical Co., Hicksville, N.Y.) [see Papsidero, L. D. et al., Hybridoma, 1: 275-82 (1982)].

No loss of McAb F36/22 binding levels was noted after 4 hours even at the highest temperature tested, 37°C. This indicated no external modulation or redistribution of McAb F36/22-complexed cell surface antigen. Immunofluorescence examination of identically-treated cells showed the presence of strong staining which was limited to the cell surface.

5.7.3. RELEASE OF ANTIGEN RECOGNIZED BY McAb F36/22 BY TUMOR CELLS

To examine if antigen recognized by McAb F36/22 was spontaneously released by tumor cells, pleural fluids were examined using a nitrocellulose direct-binding assay procedure. These fluids were obtained from patients with disseminated breast carcinoma and were histopathologically evaluated for the presence of tumor cells.

Detection of antigen which was adsorbed to nitrocellulose paper was performed using an antigen spot test as described by Herbrink et al. [J. Immunol. Methods, 48: 293-8 (1982)]. Briefly, antigen samples in the form of pleural fluids (or chromatography fractions as described in Section 5.7.4.) were applied as 10 µl drops to nitrocellulose transfer paper (BioRad, Richmond, CA) and allowed to dry for 15 minutes Thereafter the paper was inactivated by incubation in assay buffer (0.1M Tris-HCl, pH 7.4 containing 0.25% (w/v) gelatin and 0.5% (v/v) nonidet P40). The paper was then incubated for 18

hours at 4°C with antibody F36/22 at 10 µg/ml of complete culture medium. After washing the paper 4 times with assay buffer, the bound antibody was detected by incubating the paper with ^{125}I-labeled goat F(ab')$_2$ antibodies to murine immunoglobulin [Fraker, P. J. and Speck, Jr., J. C., Biochem. Biophys. Res. Commun., 80: 849-57 (1978)]. After washing, the paper was sectioned and individual samples counted for bound radioactivity.

To avoid spurious binding to human immunoglobulins, the radiolabelled anti-murine antibody was affinity-purified on murine immunoglobulin-Sepharose columns and further adsorbed with solid-phase human immunoglobulins [March, S. C. et al., Anal. Biochem., 60: 149-52 (1974)]. The resulting antibody preparation demonstrated less than 1% cross-reactivity against purified human IgG.

When compared to normal serum donors, the malignant effusions tested exhibited an increased level of McAb F36/22 binding activity and increased incidence of elevated antigen amounts (p less than 0.02); 9 out of 10 samples derived from healthy blood serum donors bound less than or equal to 3923 cpm while 17 out of 24 pleural fluid samples from breast carcinoma patients exhibited binding of 3923 cpm or more, with one sample binding as high as $1x10^5$ cpm. Also, when control murine immunoglobulin was substituted for McAb F36/22, no difference was observed between normal donors and cancer patients, indicating immunological specificity for the reaction.

Though physicochemical characterization of the pleural effusion-borne antigen(s) carrying the specific epitope recognized by McAb F36/22 were not performed as in Section 5.7.4., the results of the antigen spot test suggest serological applications for McAb F36/22.

5.7.4. GEL FILTRATION CHROMATOGRAPHY OF MCF-7 ANTIGEN RECOGNIZED BY McAb F36/22

MCF-7 primary breast carcinoma cells were homogenized at 4°C in detergent buffer (10 mM Tris, pH 7.4/0.5% (w/v) sodium deoxycholate/0.5 mM phenylmethylsulfonyl fluoride). Nuclei and debris were removed by centrifugation at 40,000xg for 1 hour. Five ml of the clear supernatant were applied to a column (2.6x100 cm) packed with Sephacryl S-300 gel filtration media (Pharmacia) and equilibrated with the above detergent buffer. The column was run at a flow rate of 15 ml/hr and fractions of 5 ml were collected. The column was pre-calibrated using protein markers of known molecular weight [thyroglobulin (19.2s), immunoglobulin G (7s), and serum albumin (4.5s)].

Fractions collected were individually tested for their immunoreactivity against monoclonal antibody F36/22 using the antigen spot test (see Section 5.7.3.). As control, the test was also performed using P3X63-Ag8 immunoglobulin as a substitute for specific antibody. Fractions eluting at approximately 5s demonstrated significant reactivity against McAb F36/22. These fractions showed no such reactivity versus control immunoglobulin.

5.7.5. EFFECT OF ENZYME DIGESTIONS ON CELL SURFACE ANTIGENICITY

Breast carcinoma cells (MCF-7) at a concentration of $1x10^6$ cells/ml were subjected to the following reagents (final concentration) at 37°C: Papain (Boehringer-Mannheim, Indianapolis, Ind.) at 0.8 U/ml in pH 6.3 PBS containing 1 mM cysteine and 1 mM EDTA; Trypsin

(GIBCO) at 1372 U/ml of Hank's salt solution containing 0.5 mM EDTA; Pepsin (Sigma, St. Louis, Mi.) at 243 U/ml of acetate buffer pH 4.5; Neuraminidase (Boehringer-Mannheim) at 0.05 U/ml of acetate buffer pH 5.0 containing 1 mM phenylmethyl sulfonyl fluoride; α-mannosidase (Sigma) at 1 U/ml of acetate buffer, pH 4.5 containing 1 mM ZnCl; Sodium-meta-periodate (Sigma) at 50 mM in PBS.

Cells were incubated with proteolytic enzymes for 5 minutes while the incubations with carbohydrate-cleaving agents were allowed to continue for 30 minutes. Cell viability was greater than 85% after each of these treatments. Subsequently, the cells were washed and assayed for residual antigenicity by direct-binding CS-RIA as described in Section 5.5. using monoclonal antibody F36/22.

The cell surface component recognized by F36/22 was labile under proteolytic conditions and resisted carbohydrate- cleaving reagents.

Direct binding of McAb F36/22 to breast carcinoma cells, and hence, cell-surface antigenicity was significantly diminished after treatment of the cells with papain and trypsin. Papain digestion and trypsin digestion reduced direct binding activity to approximately 40% and 10% of control values, respectively. Treatment with pepsin showed no effect on direct binding. All carbohydrate-cleaving agents tested produced a dramatic increase in antibody binding activity: Neuraminidase, α-mannosidase and periodate increased direct binding by 200%, 250% and 400% of control values, respectively. This effect was not based upon cellular uptake of the antibody reagents, as in each case greater than 85% viability of the cells was observed post-treatment.

The reason for the dramatic increase in antigenicity following treatment with carbohydrate-active reagents such as periodate and glycosidases may be that these reagents clear and/or "unmask" the cell surface of some carbohydrate moieties thus allowing greater access of McAb F36/22 to determinants directly adjacent to the cell membrane.

5.8. IN VITRO IMMUNOHISTOLOGICAL APPLICATIONS OF MONOCLONAL ANTIBODY F36/22

5.8.1. IMMUNOPEROXIDASE STAINING OF TUMOR SPECIMENS BY McAb F36/22

In a first set of experiments, sections of formalin-fixed and paraffin-embedded human tumor tissues were used for immunoperoxidase staining as described previously [Heyderman, E. and Neville, A.M., J. Clin. Path., 30:138-140(1976)]. Briefly, hydrated sections were treated with 20% pre-immune rabbit serum and then incubated with 100 µl F36/22 hybridoma culture fluid (7.5 µg antibody/ml) for 1 hour at room temperature. Following 2 washes in D-PBS, peroxidase-conjugated rabbit antibodies against murine immunoglobulin (1/30 dilution; Accurate Chemical Corp.) were applied for 1 hour and the slides further washed. Antibody-enzyme conjugates were pre-adsorbed with solid-phase human immnoglobulin to avoid spurious binding due to the possible presence of human globulin in tumor specimens. Enzyme activity was revealed using a diamino-benzidine/H_2O_2 substrate [Heyderman and Neville, (1976), Supra]. For some experiments, unfixed cryostat sections of fresh breast tumors were used to assess antibody reactivity.

Overall, 17 out of 22 specimens of intraductal breast carcinoma showed the presence of antigen recognized

by McAb F36/22 (TABLE IV). Several specimens of non-mammary tumors also shows immunoperoxidase staining. However, as summarized in TABLE IV, these specimens produced a weak staining intensity and/or low frequency of positive reactions. This cross-reactivity was restricted to adenocarcinomas; other tumor types (sarcoma, lymphoma, myeloma) were consistently negative. Benign lesions of the breast were further tested with the immunoperoxidase technique using F36/22; these included 3 fibroadenomas and 6 fibrocystic disease. Two specimens each showed positive staining reaction at the apical membrane portion of ductal elements. Sections obtained from normal pancreas (n=4), prostate (n=5), colon (n=3), liver (n=4), spleen (n=2) and skin (n=3) were negative, as was a specimen of DMBA-induced mammary cancer of the rat.

TABLE IV

IMMUNOPEROXIDASE STAINING OF TUMOR SPECIMENS USING MONOCLONAL ANTIBODY F36/22[a]

	Number of Specimens			
Tumor Type	Tested	Strongly Positive[b]	Weakly Positive[c]	Negative
Breast Carcinoma	22	10	7	5
Colon Carcinoma	10	1	2	7
Lung Carcinoma	3	0	1	2
Uterus Carcinoma	10	1	3	6
Liver Carcinoma	2	0	0	2
Thyroid Carcinoma	1	0	0	1
Bladder Carcinoma	6	0	0	6
Prostate Carcinoma	5	0	1	4
Pancreas Carcinoma	2	0	0	2
Lymphosarcoma	3	0	0	3
Lymphoma	2	0	0	2
Plasmacytoma	1	0	0	1
Melanoma	2	0	0	2
Astrocytoma	2	0	0	2

a. All specimens examined represent formalin-fixed and paraffin-embedded tissues examined using monoclonal antibody F36/22 at a concentration of 7.5 µg/ml.

b. Greater than 10% of the tumor cells exhibiting intense cytoplasmic staining.

c. Less than 10% of the tumor cells exhibiting staining or exhibiting a weak staining reaction limited to the luminal surface of ductal elements.

In an expanded investigation of the in vivo tissue distribution of the epitope recognized by McAb F36/22, immunoperoxidase staining was performed on extra-mammary tumor specimens using a variation of the technique described supra.

Paraffin-embedded blocks of tissue were obtained from the Pathology Departments of St. Joseph's Intercommunity Hospital and Roswell Park Memorial Institute, Buffalo, New York. All tissues were fixed immediately in 10% buffered formalin, embedded in paraffin, and sectioned at 3 to 4 µm for these studies. The sections were collected on slides pretreated with ovalbumin and glycerine and heated at 70° C for 5 minutes to increase the adherence of the sections to the glass slides.

Hydrated sections were treated with 10% preimmune rabbit serum in 1% ovalbumin and then incubated with 100 µl of hybridoma culture fluid (10 µg antibody/ml) for 90 minutes at room temperature. Following washes in D-PBS containing 0.01% Nonidet P-40, peroxidase-conjugated rabbit antibodies against murine immunoglobulin (1/30 dilution; Accurate Chemical Corp,1) were applied 45 minutes. Human immunoglobulin, convalently-linked to Sepharose beads was used to pre-adsorb the antibody-enzyme conjugate in order to prevent binding to human

immunoglobulin that might be present in the tumor specimens. In most cases, enzyme activity was revealed using diamino-benzidine/H_2O_2 substrate in phosphate buffer, pH 6.3. In very sanguinous tissues which have large amounts of endogenous peroxidases, amino-ethyl-carbazole (4 mg/ml; Sigma)/ H_2O_2 was used as the substrate to better ascertain specific binding. In addition to the normal control substitution experiments, which included PBS in place of the primary antibody, peroxidase-conjugated antibody alone and culture fluid from a myeloma cell line (FLOPC-21), which secreted an irrelevant IgG3 antibody, were used. The intensity of the immunoreaction product was scored using a 0 to +++ scale for TABLE V, however, in order to facilitate statistical evaluations of the staining intensity data, corresponding numerical values of 0 to 3 were utilized.

The results of immunoperoxidase staining on a large group of extramammary tumors are shown in TABLE V. A varying incidence of antigen expression was observed for these specimens. The majority (54/58) of immuno-positive tumors were histologically classified as adenocarcinomas and no detectable antigen was expressed by other carcinoma types, including basal cell carcinoma, bronchogenic carcinoma (squamous, large cell and oat cell), squamous cell carcinomas of the rectum and endocervix, malignant melanocarcinoma, esophageal carcinoma (squamous and adenocarcinoma) and squamous cell carcinoma of the bladder. The other 4 positive tumors were transitional cell tumors of the bladder and renal pelvis. The predominant location of staining in the majority of these positive tumors was the apical luminal surface of the epithelial cells, although the intensity of staining was usually lower than that observed for breast carcinomas (TABLE V).

For several carcinoma sites, including colon, pancreas, jejunum, stomach, prostate and ovary, the adjacent tissue was consistently negative for expression of antigenic determinant, but the tumor was positive. Of additional interest, a few specimens of pancreas and colon carcinomas showed expression of the antigenic determinant in the epithelial elements of the adenocarcinoma and also in apparently normal tissue directly adjacent to the carcinoma, whereas normal tissue further distal to the tumor was negative. No detectable reactivity with a large panel of tumors of mesenchylmal origin was observed (TABLE V), including lymphosarcomas, myelomas, astrocytomas, leukemias and sarcomas.

TABLE V

IMMUNOPEROXIDASE STAINING OF EXTRA-MAMMARY TUMORS WITH McAb F36/22

Tissue Examined	No. Staining/ No. Examined	Predominant Location of Stain[a]	Intensity of Stain[b]	$\bar{x}$% Positive cells (range)[c]
Gastric Carcinoma	5/10	S	+ to ++	40 (10-60)
Jejunal Carcinoma	1/2	S	+	30
Colonic Carcinoma	3/9	S	+ to ++	60 (50-80)
Cholangic Carcinoma	6/7	S	+ to ++	60 (30-90)
Pancreatic Carcinoma	4/10	S	+	40 (10-90)
Thyroid Carcinoma	1/2	S	+	40
Endocervical Carcinoma	1/2	S	+ to ++	80
Endometrial Carcinoma	9/13	S/C	+ to +++	70 (30-90)
Fallopian Tube Carcinoma	0/2			
Ovarian Carcinoma	15/15	S	+ to +++	80 (70-90)
Prostatic Carcinoma	2/8	S	+	10 (20-20)
Renal Carcinoma	3/6	S	+ to +++	40 (20-60)
Bronchogenic Carcinoma	5/13	S/C	+ to +++	70 (50-90)
Ovarian Cystadenoma	3/3	S/C	+ to +++	40 (20-80)
Bladder Carcinoma	2/5	C	++	30 (20-40)

a. S=Marginal and/or luminal surface; C=cytoplasmic; S/C = surface and/or cytoplasmic.

b. Intensity scale: - negligible; + weak; ++ moderate; +++ strong.

c. Percent of positive staining cells was estimated by observing the number of positive cells in replicate 100 x fields.

d. No staining was observed in the following extra-mammary tumor specimens (the number in parenthesis indicates the number of samples examined): esophageal carcinoma (5); hepatic carcinoma (2); parotid gland carcinoma (3); submaxillary gland. carcinoma (1); seminoma (2); malignant melanoma (3); basal cell carcinoma (4); squamous cell carcinoma (3); sebaceous gland carcinoma (1); leukemias (4); multiple myeloma (3); meningioma (2); astrocytoma (3); lymphosarcoma (6); Kaposi's sarcoma (1); fibrosarcoma (3); glomus tumor (1); carcinoid (2); teratoma (4); hidroadenoma (4); myoxma (3); neuroma (3); hemangioma (2); hamartoma (2); lymphangioma (1); lipoma (4); trichoepithelioma (3); fibroxanthoma (2); nevus (benign) (1);

5.8.2. IMMUNOREACTIVITY OF McAB F36/22 WITH NORMAL MAMMARY TISSUE, MEMBRANE PREPARATIONS AND MILK

McAb F36/22 was tested for its ability to react with mammary membranes using adsorption analysis and direct binding radioimmunassay procedures. To obtain tissue membranes, normal breast tissue was homogenized in D-PBS containing protease inhibitors (0.5 mM PMSF and 1.0 mM epsilon-aminocaproic acid). All steps were performed at 4°C. Cell debris and nuclei were pelleted at 2,000 xg and the supernatant was further centrifuged at 40,000 xg to obtain a crude membrane fraction. The membranes were washed three times with D-PBS and adjusted to 2 mg protein/ml.

Membranes from human milk were isolated as follows: the cream fraction was washed 5 times with D-PBS to remove whey proteins and was quickly frozen. The cream was then slowly thawed, thereby increasing the rupture of membrane globules, and then was shaken vigorously in D-PBS containing 1 mM $MgCl_2$. When butter formation was observed, the suspension was centrifuged at 100,000 xg for 1 hour. Membranes were resuspended in D-PBS at 2 mg protein/ml.

For direct binding measurements, 50 µl samples of membrane preparations were added to wells of glutaraldehyde-sensitized microtiter plates. After adhering overnight, the wells were washed twice with 0.25% glycine and then 4 times with D-PBS containing 1% bovine serum albumin. The assay was performed in triplicate using 100 µl samples of McAb F36/22 which were incubated with the membranes for 90 minutes at room temperature. The remainder of the assay was performed as in Section 5.5., for CS-RIA procedure.

Membrane fractions obtained by the foregoing procedures from both tissues and human milk fat globules were examined along with the frozen sections of normal mammary tissues. As shown in TABLE VI, antibody F36/22 directly bound to all membrane preparations examined, and was able to be adsorbed by these specimens. Immunoperoxidase data confirmed the identity of the reactive antigen as a component of the ductal epithelial surface membrane.

TABLE VI

IMMUNOREACTIVITY OF NORMAL MAMMARY TISSUES AND MEMBRANE PREPARATIONS

Specimen	Reactivity with Antibody F36/22
Mammary Tissues[a]	+
Membranes[b]	
Tissues	+
Milk Fat Globule	+

a. Cryostat sections of frozen mammary tissues (n=3) were examined by standard immunoperoxidase staining techniques (see Section 5.8.1).

b. Membrane preparation at 2 mg protein/ml were tested for their immunoreactivity using absorption analysis and direct-binding radioimmunoassay (see Section 5., and 5.5., respectively).

A second set of experiments testing the reactivity of McAb F36/22 with normal tissues was performed using the immunoperoxidase staining technique. The results are summarized in TABLE VII.

In general, positive staining was restricted to the epithelial elements of a few exocrine glands and their associated ducts. These included sweat glands, sebaceous glands, and endometrial glands. Other tissues with positive staining were respiratory alveoli, ductuli efferentes of epididymis, fallopian tube and the distal tubules and collecting ducts of the kidney. These tissues exhibited a very weak, apical luminal staining.

There was no relationship between the degree of positive staining in endometrial glands and the different phases of the menstrual cycle. Additionally, the tissue components of ovary, colon, stomach, pancreas, prostate, and gall bladder did not contain detectable levels of the antigen determinant recognized by McAb F36/22, whereas the adenocarcinomas of these nistotypes did express detectable levels to a varying incidence. The bile ducts of liver, the acinar cells of the pancreas, and the serous demilunes of the salivary gland were all negative for the expression of the epitope.

TABLE VII

IMMUNOPEROXIDASE STAINING OF NORMAL TISSUE WITH McAb F36/22[a]

Positive Staining Restricted to Epithelial Elements of:
The globules and cell surfaces of sebaceous glands (5/5)[b], alveolar epithelium of lung (4/5), sweat glands and associated ducts (5/5), ductuli efferentes of epididymis (3/3), endometrial glands and ducts (8/8), fallopian tube (4/5), distal tubules and collecting ducts of kidney (4/4).

No Staining of Tissue Elements of:

Esophagus (2)[c], stomach (3), small intestine (3), appendix (3), colon (5), pancreas (4), gall bladder (2), liver (5), parotid gland (2) submaxillary gland (3), thyroid (3), adrenals (2), spleen (3), tonsil (2), lymph node (2) bone marrow (3), prostate (5), ovary (15), testes (4), nervous tissue (5), skin (5), myocardium (3), trachea (1) and bladder (2).

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a. Normal tissues distal to tumors examined in the present study were also evaluated for expression of this antigen determinant, but these data were not included in the above numbers.

b. No. of tissues staining/No. of tissues examined.

c. The No. in parentheses represents total No. examined.

5.8.3 IMMUNOPEROXIDASE STAINING OF HUMAN BREAST TISSUES AND TUMORS

Non-malignant and primary breast tumor specimens were obtained at surgery. Freshly obtained autopsy specimens of normal tissues and distant metastatic lesions were also examined. All tissues were fixed immediately in 10% buffered formalin, embedded in paraffin, and sectioned at 3 to 4 µm for these studies. The sections were collected on slides pretreated with ovalbumin and glycerine and heated at 70°C for these studies. The sections were collected on slided pretreated with ovabumin and glycerine and heated at 70° for 5 minutes to increase adherence of the sections to the glass slides. The immunoperoxidase staining technique described for the expanded investitgation in Section 5.8.1. was employed.

The results of tissue staining for normal resting breast, benign and malignant breast tumors are presented in TABLE VIII. In general, the normal resting breast parenchyma exhibited delicate, but weak, immunoperoxidase staining restricted to the apical luminal surface of epithelial cells. Benign tumors of the breast displayed similar location of the staining reaction, except that the intensity of the reaction was generally greater. All gynecomastia specimens examined showed positive immuno-reactivity consistent with that observed in the benign conditions. Primary breast tumors, however, displayed significant variations in the percentage of cells stained in any given tumor, in the intensity of the staining reaction and, and in the location of the immuno-reaction. As is summarized in TABLE VIII, expression or lack of expression of the antigenic determinant did not correlate with the grade of the tumor. However, McAb F36/22 predominantly stained the surface membranes of luminal epithelial cells in well-differentiated carcinomas and those cancer cells bordering lumina within the in situ and comedo tumors. Poorly-differentiated tumors, when stainable, most often exhibitd focal cytoplasmic staining. Intensity of the reaction product varied from specimen to specimen (TABLE VIII).

TABLE VIII

INDIRECT IMMUNOPEROXIDASE STAINING OF HUMAN BREAST TISSUE WITH McAb F36/22

Tissue Examined	No. Staining/ No. Examined	Predominant Location of Stain[a]	Intensity of Stain[b]	$\bar{x}$% Positive cells (range)[c]
Normal				
Resting Breast	4/4	S	+	70(40-90)
Non Malignant Lesions				
Fibrocystic disease	6/6	S	+ to +++	70(40-90)
Fibroadenoma	6/6	S	+ to +++	80 (70-90)
Cystosarcoma Phylloides	1/1	S	+++	90
Papilloma	2/2	S	+ to ++	90(80-100)
Gynecomastia	5/5	S	+ to +++	70 (20-90)
Malignant Lesions				
Ductal Carcinoma:				
Tubular type	0/2			
Moderately diff.	4/4	S/C	+ to +++	30 (20/50)
Poorly diff.	10/13		+ to +++	70 (30-90)
Comedo type	5/5	S/C	+ to +++	80 (50-90)

Tissue Examined	No. Staining/ No. Examined	Predominant Location of Stain[a]	Intensity of Stain[b]	$\bar{x}$% Positive cells (range)[c]
Infiltrating Ductal Carcinoma:				
Well diff.	3/4	S	+ to +++	50 (40-80)
Well-Mod. diff.	1/1	S/C	+++	90
Moderately diff.	7/10	S/C	+ to +++	60 (10-90)
Mod.-Poorly diff.	8/9	S/C	+++	90(80-100)
Poorly diff.	17/18	C	+ to +++	60 (40-90)
Mucoid Carcinoma	3/3	S/C	++ to +++	90(80-100)
Lobular Carcinoma	8/9	C	+ to +++	80(50-90)
Medullary Carcinoma	1/1	C	++ to +++	60
Squamous Cell Carcinoma	1/1	C	+ to ++	50
Lymphosarcoma	0/1			
Metastatic				
Lymph nodes	5/12	S/C	+ to +++	50 (30-90)

a. S=marginal and/or luminal surface; C=cytoplaasmic; S/C = surface and/or cytoplasmic.

b. Intensity scale: - negative; + weak; ++ moderate; +++ strong.

c. Percent of positive staining wells was estimated by observing replicate 100x fields of view.

5.8.4. EFFECT OF VARYING McAb F36/22 CONCENTRATION AND INCUBATION TIMES ON IMMUNOPEROXIDASE STAINING RESULTS

To determine if the lack of detectable antigen expression in some tumors was merely reflecting an inadequate antibody concentration, the following study was performed. Two reference breast carcinomas which were known to be positive for the expression of the epitope recognized by McAb F36/22 were serially sectioned. Serial dilutions of McAb F36/22 (initial concentration: 10 µg/ml) were incubated with the sections which then were evaluated by immunoperoxidase staining. The highest dilution which exhibited immunopositivity when compared to the initial concentration was determined.

Increasing the concentration of McAb F36/22 from 10 to 20 µg/ml did not significantly increase the percentage of tumors exhibiting positive immunoperoxidase staining. Furthermore, antibody at 200 ng/ml was capable of staining greater than 95% of the original immunopositive cells. Thus, the present experiments were performed at antibody excess. Increasing the incubation times also did not affect the sensitivity of the assay.

5.8.5. ESTROGEN RECEPTOR LEVELS AND IMMUNOPEROXIDASE STAINING

To determine if the staining patterns recognized by McAb F36/22 were related to estrogen receptor status, 60 breast tumors of known estrogen receptor levels were stained by immunoperoxidase techniques as previously outlined. The estrogen receptor levels of these specimens were determined at the Buffalo General Hospital, Department of Clinical Chemistry, as described by Rosen, P.P. et al. [Cancer Res. 35:3187-3194(1975)].

Breast cancer specimens of known estrogen receptor level (n=60) 42 ER-rich, 18 ER-poor, were examined by immunoperoxidase techniques. The percentage of positive cells, the mean ± S.D., (69±30) in the ER-rich carcinomas was greater (p less than .001) than that exhibited in ER-poor cancers (33± 37). In addition, the intensity of the staining reaction associated with ER-rich cancers (2.1±0.8) was significantly greater (p less than .001) than the reaction product observed in ER-poor carcinomas (1.0±0.9), as assessed using the semi-quantitative technique of immunoperoxidase staining.

5.9. IN VIVO APPLICATIONS OF MONOCLONAL ANTIBODY F36/22

5.9.1. EXPERIMENTAL INDUCTION OF SOLID TUMORS IN MICE

Human tumor cells were mechanically-harvested from tissue culture flasks and washed with serum-free RPMI-1640 medium. Cell preparations showing greater than 90% viability, as assessed with trypan blue, were used to produce solid tumors in mice. Athymic female Swiss nude (nu/nu) mice were given subcutaneous injections of 0.2 ml containing 4-10x10^6 viable tumor cells. MCF-7 and BT-20 (human breast carcinoma), Chago (human lung carcinoma) and Daudi (lymphoblastoid cells) were used. Tumor dimensions were subsequently monitored at daily intervals using calipers and the tumor volume was caluclated using a formula described by Kovnat. A. et al. [Cancer Res., 42: 3969-73(1982)].

5.9.2. McAb F36/22 TARGETING: IN VIVO TUMOR LOCALIZATION

To evaluate whether immunotherapeutic effects could be attributed to a direct interaction between tumor and antibody, targeting experiements were performed to

determine the tissue distribution of injected radio-antibody.

For one set of antibody targeting experiments, purified immunoglobulins were labelled with iodine-125 to a specific activity of 5 Ci/mo] [Fraker, P.J. and Speck, Jr., J.C., Biochem. Biophys. Res. Commun., 80:849-57(1978)]. Athymic mice bearing human tumor xenografts induced by MCF-7 or Chago cells, were given a single intraperitoneal injection of radiolabelled immunoglobulin (10-20 µCi) on day 0. On day 7, the animals were exsanguinated by cardiac puncture and tumors and organs were removed. After weighing each tissue, radioactivity was measured in a well-type gamma counter. The results were expressed as a localization ratio, i.e., cpm per gram of tissue (tumor or organ) divided by cpm per gram of blood.

As shown in TABLE IX, McAb F36/22 localized to breast tumors in greater amounts per gram of tissue than other organs examined. Effective targeting of radio-antibody to breast tumor xenografts indicates a direct interaction between tumor cells and F36/22 monoclonal antibody. This targeting effect was observed with the use of control immunoglobulin preparations or against lung carcinoma xenografts (TABLE IX).

TABLE IX

TARGETING OF RADIOLABELLED MONOCLONAL ANTIBODY (F36/22) IN ATHYMIC MICE BEARING HUMAN TUMOR XENOGRAFTS[a]

Tissue[b]	Localization Ratio[c]
Blood	1
Breast Carcinoma (MCF-7)	3.4 ± 0.5
Lung Carcinoma (Chago)	0.7 ± 0.1
Lung	1.2 ± 0.3
Liver	0.5 ± 0.2
Spleen	0.4 ± 0.1
Kidney	0.5 ± 0.2
Brain	0.5 ± 0.1

a. Athymic mice (4 per group) were each implanted with 2 human tumor xenografts on contralacteral sites of the back. These consisted of breach (MCF-7) and lung (Chago) carcinomas. Xenografts were of similar size at onset of the experiments (between 5 and 15 mm^3). Each animal received between 10 to 15 µCi of radiolabelled monoclonal antibody (F36/22) by the intraperitoneal route.

b. Tissues and blood were harvested 7 days post injection.

c. Localization Ratio: cpm per gram tissue (tumor or organ) divided by cpm per gram of blood, (mean ± S.D.).

In a second set of targeting experiments, BT-20 (human breast carcinoma), and Chago (human lung carcinoma) were used. The growth pattern of the xenografts was carefully monitored in order to consistently work with similar sizes of growing tumors. In vivo tumor localization was performed essentially the same as above. Twenty micrograms of ^{125}I-labelled McAb F36/22 (specific activity, 1μCi/μg) was injected intraperitoneally using four groups of nude mice (n=3) bearing either BT-20 or Chago tumors. One group of mice was sacrificed at sequential days after administration of radiolabelled McAb F36/22. Twelve tissues were removed (blood, liver, lung, spleen, heart, skeletal muscle, skin, kidney, intestine, brain, and tumor), and the tissue distribution of the labelled antibody was determined with a Gamma counter. Tissue levels were calculated in disintegrations per minute per gram of tissue. Blood levels were taken as the reference backgroud so that relative antibody uptake into tissues (localization ratio) was determined to be the ratio of disintegrations per minute per gram of tissue divided by the disintegrations per minute per gram of blood. The uptake of McAb in the BT-20 tumors was 5.7 ($\pm$0.4), (mean $\pm$ standard deviation) fold greater than the blood background (p less than 0.02), at 7 days post injection; whereas no other tissue including Chago tumors showed statistically significant activity above blood levels. In contrast, normal mouse ^{125}I-labelled antibody (γ3 subclass) showed no specific uptake into any tissues or tumors sampled. Significant specific uptake of McAb F36/22 was also seen in MCF-7 breast carcinoma, which gave a localization ratio of 3.4 ($\pm$ .5), as compared to blood levels (p less than 0.05).

5.9.3. IN VIVO PASSIVE IMMUNOTHERAPY WITH McAb F36/22

For one set of therapy studies, mice with progressively growing human tumors (MCF-7, Chago or Daudi) were given a single intraperitoneal injection at day 0 of either monoclonal antibody F36/22 or control immunoglobulin. One hundred micrograms were administered in a total volume of 0.2 ml sterile PBS. Mean tumor volumes at day 0 were: MCF-7,57 cu. mm.; Daudi, 59 cu.mm; Chago, 105 cu.mm. Post-therapy tumor volumes were taken at daily intervals and statistical evaluations were performed using Student's t-test. Based upon these measurements the tumors were scored as either progressive, static, or regressive. For each experiment mice were monitored for one week, at which time tumor specimens were excised for histological examination. Results obtained using caliper measurements indicated that breast carcinoma xenografts treated with antibody F36/22 had regressed to approximately 15% of their pre-treatment volumes (57 to $7mm^3$) at day 7. As expected with the use of a murine host, non-immune mouse IgG produced no such efect at the same dose level. The therapeutic effect of antibody F36/22 was evident by day 4 post-therapy, when a significant (p less than 0.01) decrease (approximately 75%) in breast tumor volumes was observed. Replicate groups of antibody-treated mice which were observed for 2 weeks demonstrated breast tumor xenografts exhibiting no detectable capacity for regrowth during this period. Histological examination of breast tumors obtained 7 days post-therapy indicated a significant amount of tumor cell necrosis accompanied by initial stages of fibrosis. In contrast, the histological picture of control tumors treated with non-immune IgG was consistent with the normal histology of MCF-7 breast carcinoma xenografts.

The therapeutic specifity of antibody F36/22 was evaluated against two non-mammary human tumor xenografts implanted in nude mice. For these experiments a slow growing lymphoma (Daudi) and rapidly multiplying lung carcinoma (Chago) were used. Results indicate that antibody treatment was ineffective in causing regression of these tumors within 4 days post-therapy. Further, at 7 days post-therapy, the lymphomas were at over 300% of their day 0 volume (from 59 to 222 mm^3 at days 0 and 7, respectively). A similar progression of lung carcinoma xenografts was also observed (105 and 706 mm^3 at days 0 and 7, respectively).

The effects of McAb against tumors in vivo were tested in another experiment. One group of mice (n=4), bearing human breast carcinoma (BT-20) were inoculated intraperitoneally with 100 µg of purified [Ey, P.L., et al; Immunochem., 15:429-436 (1978)] McAb F36/22. A significant reduction (40%) of tumor volumes was seen for each of the individual mice one day following injection of McAb F36/22 (p less than 0.001 for each tumor as compared to pre-treatment volumes). Tumor volumes were measured daily with precision caliper and calculated by the 3 diameter product. No tumor volume reduction occurred during the 7 days following administration of the control antibody.

In order to test reproducibility and statistical significance of the McAb therapy, further experiments were performed. After 3 weeks to 2 months post-implantation, 26 BT-20 breast tumors of sizes ranging from 12 to 199 mm^3 (mean, 61mm^3) were produced. These tumor-bearing mice were injected with either 100 µg of purified McAb F36/22 or one of 2 identically-purified control antibodies (one a γ3 myeloma protein with no known specificity, the

other a γ2a complement-fixing McAb with no cell binding specificity to BT-20 cells). Fourteen mice with tumors ranging from 18 to 199 mm^3 (mean±SD, 62±46mm^3) were each injected intraperitoneally with 100 µg of McAb F36/22 in PBS, and 12 mice with tumors ranging from 12 to 149 mm^3 (mean±SD, 61±42 mm^3) with the same amount of control antibody (7 of the control mice with the γ3 protein and 5 of the control mice with the γ2a McAb). Therapy of breast cancer with McAb F36/22 resulted in a rapid reduction in tumor size. Tumor regression continued for about 3-4 days post-therapy and thereafter the tumors remained at approximately 25 percent of their pretreatment volume. In comparison, breast tumors were at 115 (± 37) percent of original volume after treatment with control immunoglobulin. Measurements of the control antibody-treated group showed 6 mice had slowly enlarging tumors; 2 tumors were stable and 4 tumors were slowly regressing in volume over the 7 days of the experiment. Similar variations of growth patterns were typically seen in breast carcinoma xenografts as thoroughly described by others. [Ozzelo, L. and Sordat, M; Eur. J. Cancer; 164:553-559 (1980)]. The McAb F36/22 produced no regression of Daudi lymphoid tumors or fast growing Chago lung carcinomas. The BT-20 tumors were significantly reduced in volume (p less than 0.001) as compared to controls at 24 hours post-therapy and remained at a decreased volume for the course of the 7 day experiment. The mice with closely matched tumor sizes were segregated into groups consisting of small and large BT-20 tumors, in order to judge the possible influence of pre-treatment tumor size on the amount of tumor volume reduction following therapy. The first group consisted of 11 mice with tumors ranging from 26-49 mm^3. The second group had 9 mice with tumor volumes ranging from 60-88 mm^3. The smaller tumors decreased to 5(±3) mm^3, 4 days after

McAb F36/22 treatment as compared to 51(±25) mm^3 for control antibody treated mice (p less than .001) (TABLE X). The larger tumors were reduced to 25(±18) mm^3 4 days after McAb F36/22 therapy, as compared to 74(±20) mm^3 for controls (p less than 0.005) (TABLE X). No statistically significant difference was observed in the percentage of tumor volume reduction between the larger and smaller tumor groups.

Tumor histology which was taken before therapy and at 1,3 and 5 day intervals after the injection with 100 µg of McAb F36/22, demonstrated extensive necrosis of tumor cells on days 1,3, and 5. On day 1, tumor cell necrosis was most prominent in the central region of the tumor, leaving a semi-circular ring of intact tumor cells (1 to 20 cells thick) at the periphery of the tumor. Acute inflammatory cell infiltration was also seen. By day 3, tumor contraction was observed, accompanied by both chronic and acute inflammatory cells. On day 5 scar tissue activity was evident along with a predominantly chronic inflammatory pattern of cell infiltration. These histological changes appear to be very similar to those observed by others using a polyclonal sera on a virally infected chemically induced murine sarcoma [Ward, E.W. et al., J. Natl. Cancer Inst. 69:509-515 (1982)]. Extensive tumor cell necrosis was also seen for MCF-7 breast tumors treated with McAb F36/22 and no histological changes were seen in identically treated Chago tumors as compared to control tumors.

It is apparent that many modifications and variations of this invention as hereinabove set forth may be made without departing from the spirit and scope thereof. The specific embodiments described are given by way of example only and the invention is limited only by the terms of the appended claims.

A cell line, F36/22, as described herein has been deposited with the American Type Culture Collection, Rockville, Maryland, and has been assigned accession number ATCC No. HB8215. The invention described and claimed herein is not to be limited in scope by the cell line deposited, since the deposited embodiment is intended as a single illustration of one aspect of the invention and any equivalent cell lines which produce a functionally equivalent monoclonal antibody are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

TABLE X

COMPARISON OF PRE-TREATMENT TUMOR SIZE TO McAb F36/22 MEDIATED VOLUME REDUCTIONS[a]

Antibody	Number of Mice/Group	Day 0 Range of Tumor Volume (mm)	Tumor Volumes[b] Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Group I (small tumors)										
F36/22	6	26-42	34 ± 7	11 ± 8	9 ± 6	9 ± 5	5 ± 3	7 ± 3	6 ± 1	9 ± 4
Control (γ3)	5	25-49	38 ± 8	39 ± 2	46 ± 14	44 ± 16	51 ± 25	48 ± 30	45 ± 26	41 ± 20
Level of significance (less than)	-	-	N.S.[d]	.001	.001	.001	.001	.001	.001	.001
Group 2 (large tumors)										
F36/22	6	60-80	74 ± 11	33 ± 16	27 ± 15	24 ± 20	25 ± 18	22 ± 16	22 ± 18	20 ± 17
Control (γ3)	3	80-88	84 ± 4	84 ± 6	85 ± 8	81 ± 7	74 ± 20	72 ± 15	69 ± 10	70 ± 14
Level of significance (less than)	-	-	N.S.[d]	.001	.001	.001	.001	.001	.001	.001

a. Mice with BT-20 tumors were pooled separately into groups of small and large tumors, the smaller tumors ranging from 26-49 mm^3, the larger tumors 60-88 mm^3. Each group was treated with 100µg of McAB F36/22 for the experimental mice or 100 µg of control immunoglobulin for the control mice.

b. All tumor volumes given as x±SD, in mm^3.

c. p values calculated by Student's t distribution.

d. N.S., no statistically significant difference.

WE CLAIM:

1. A monoclonal antibody produced by a hybridoma formed by fusion of a myeloma cell and a cell capable of producing antibody against a tumor antigen, which monoclonal antibody is capable of shrinking tumors in vivo.

2. A monoclonal antibody produced by a hybridoma formed by fusion of a myeloma cell and a cell capable of producing antibody against a tumor antigen, which monoclonal antibody is capable of binding to and immunologically staining adenocarcinoma tissue or cells.

3. The monoclonal antibody of claim 2, which further:

a) reacts with an unmodulated surface antigen of tumor cells.

b) is capable of cytolytically fixing human complement; and

c) localizes tumors or tumor cells in vivo.

4. The monoclonal antibody of claim 3, wherein the unmodulated surface antigen is a 5s membrane component sensitive to proteolytic reagents.

5. The monoclonal antibody of claim 3, which is further capable of shrinking tumors in vivo.

6. The moncolonal antibody of claim 3, which further exhibits enhanced ability to bind tumor antigen after treatment of tumor cells with carbohydrate reactive agents.

7. The monoclonal antibody of claim 2, which is further capable of binding to and immunologically staining well-differentiated and poorly-differentiated adenocarcinoma tumors.

8. The monoclonal antibody of claim 7, wherein the adenocarcinoma tumors are human breast tumors.

9. The monoclonal antibody of claim 7, which exhibits a surface staining pattern for well-differentiated adenocarcinoma tumors and a cytoplasmic staining pattern for poorly-differentiated adenocarcinoma tumors.

10. The monoclonal antibody of claim 2, which is further capable of differentiating adenocarcinomas from normal tissue of ovarian, uterine, colonic, pancreatic or prostatic histotypes.

11. The monoclonal antibody of claim 3, wherein the tumor antigen is present on breast carcinoma cells and absent in bone marrow stem cells or lymphoid cells.

12. The monoclonal antibody of claim 1, 2, or 3 which is of class IgG.

13. The monoclonal antibody of claim 1, 2, or 3 which is of subclass IgG_3.

14. The monoclonal antibody of claim 1 or 5, wherein the shrinkable tumors are breast solid tumors.

15. The monoclonal antibody of claim 1 or 5, wherein the shrinkable tumors are adenocarcinomas.

16. The monoclonal antibody of claim 1, 2, or 3 wherein the cell capable of producing antibody against a tumor antigen is a) derived from an animal immunized with human adenocarcinoma cells; and b) is selected from the group consisting of spleen cells, lymph node cells, and peripheral blood lymphocytes.

17. The monoclonal antibody of claim 1, 2, or 3 wherein the cell capable of producing antibody against a tumor antigen is a) derived from an individual with adenocarcinoma; and b) is selected from the group consisting of peripheral blood lymphocytes, spleen cells or lymph node cells.

18. The monoclonal antibody of claim 16 wherein the human adenocarcinoma cells are breast carcinoma cells.

19. The monoclonal antibody of claim 16 wherein the human adenocarcinoma cells are ovarian carcinoma cells.

20. The monoclonal antibody of claim 18, wherein the breast carcinoma cells are MCF-7 cells.

21. The monoclonal antibody of claim 18, wherein the breast carcinoma cells are BT-20 cells.

22. The monoclonal antibody of claim 1, 2, or 3 wherein the myeloma cell is a mouse myeloma cell.

23. The monoclonal antibody of claim 1, 2, or 3 wherein the myeloma cell is P3x63Ag8.653.

24. A monoclonal antibody of class IgG produced by a hybridoma formed by fusion of a P3x63Ag8.653 myeloma cell and a lymphocyte capable of producing antibody against MCF-7 human breast carcinoma cells, which monoclonal antibody:

a) is capable of shrinking adenocarcinoma tumors in vivo;

b) reacts with well-differentiated and poorly-differentiated human breast tumors;

c) differentiates adenocarcinomas from normal tissue of ovarian, uterine, colonic, pancreatic or prostatic histotypes;

d) reacts with MCF-7, BT-20, MDA-MB-157 and SK-BR-3 breast carcinoma cell lines but not with bone marrow stem cells or lymphoid cells;

e) reacts with an unmodulated 5s surface tumor antigen sensitive to papain or trypsin digestion and resistant to periodate, neuraminidase, and α-mannosidase;

f) is capable of cytolytically fixing human complement; and

g) localizes adenocarcinoma tumors in vivo.

25. The monoclonal antibody of claim 24, produced by a hybridoma formed by fusion of a P3x63Ag8.653 myeloma cell and a splenocyte from a Balb/c mouse previously immunized with MCF-7 human breast carcinoma cells.

26. The monoclonal antibody of claim 24, produced by a hybridoma formed by fusion of a P3x63Ag8.653 myeloma cell and a splenocyte from a Balb/c mouse previously immunized with a 5s unmodulated, protease-sensitive tumor antigen isolated from the surface of breast carcinoma cells.

27. The monoclonal antibody of claim 24, wherein the lymphocyte capable of producing antibody against MCF-7 human breast carcinoma cells is derived from an individual with breast cancer.

28. A process for producing monoclonal antibodies which comprises harvesting antibodies which a) are capable of shrinking adenocarcinoma tumors in vivo; b) react with well-differentiated and poorly-differentiated human breast tumors; c) differentiate adenocarcinomas from

normal tissue of ovarian, uterine, colonic, pancreatic or prostatic histotypes; d) react with MCF-7, BT-20, MDA-MB-157 and SK-BR-3 cell lines but not with bone marrow stem cells or lymphoid cells; e) react with an unmodulated 5s surface tumor antigen sensitive to papain or trypsin digestion and resistant to periodate neuraminidase and α-mannosidase; f) are capable of cytolytically fixing human complement; and g) localize adenocarcinoma tumors in vivo, produced by a hybridoma formed by fusing a lymphocyte capable of producing anti-adenocarcinoma antibodies and a P3x63Ag8.653 myeloma cell.

29. The process of claim 28, wherein said monoclonal antibodies are harvested from in vitro cultures of the hybridoma.

30. The process of claim 28, wherein said monoclonal antibodies are harvested from ascites fluid or sera of mice injected with the hybridoma for in vivo propagation.

31. Anti-adenocarcinoma antibodies produced by the method of claim 29 or 30.

32. A continuous cell line which produces a monoclonal antibody capable of shrinking tumors in vivo, which comprises: a hybridoma of a lymphocyte derived from a mouse immunized with cells bearing tumor antigens and a mouse myeloma cell.

33. A continuous cell line which produces monoclonal antibodies capable of shrinking solid tumors associated with human breast in vivo, which comprises: a hybridoma of a lymphocyte derived from a mouse immunized with adenocarcinoma cells or an immunogenic determinant thereof and a mouse myeloma cell.

34. A continous cell line which produces monoclonal antibodies capable of shrinking adenocarcinomas in vivo, which comprises: a hybridoma of a lymphocyte derived from a human with adenocarcinoma and a myeloma cell.

35. A continuous cell line which produces monoclonal antibodies which a) are capable of shrinking adenocarcinoma tumors in vivo; b) react with well-differentiated and poorly-differentiated human breast tumors; c) differentiate adenocarcinomas from normal tissue of ovarian, uterine, colonic, pancreatic or prostatic histotypes; d) react with MCF-7, BT-20, MDA-MB-157 and SK-BR-3 breast carcinoma cell lines but not with bone marrow stem cells or lymphoid cells; e) react with an unmodulated 5s surface tumor antigen sensitive to papain or trypsin digestion and resistant to periodate, neurmaminidase, and α-mannosidase; f) are capable of cytolytically fixing human complement; and g) localize adenocarcinoma tumors in vivo, which comprises: a hybridoma of a lymphocyte derived from a mouse immunized with adenocarcinoma cells or an immunogenic fragment thereof and a mouse myeloma cell.

36. The cell line of claim 33, 34, or 35 wherein the adenocarcinoma is breast carcinoma.

37. The cell line of claim 33 or 35 wherein the adenocarcinoma cells are MCF-7, BT-20, MDA-MB-157 or SK-BR-3 breast carcinoma cells.

38. Hybridoma cell line F36/22.

39. Moncolonal antibody produced by hybridoma cell line F36/22.

40. The monoclonal antibody of claim 1, 5, or 39, conjugated to a cytotoxic agent.

41. The monoclonal antibody of claim 40, wherein the cytotoxic agent is a radioactive compound.

42. The monoclonal antibody of claim 40, wherein the cytotoxic agent is an antibiotic or antimetabolite selected from the group consisting of daunomycin, adriamycin, chlorambucil and methotrexate.

43. The monoclonal antibody of claim 40, wherein the cytotoxic agent is a cell-surface active agent selected from the group consisting of diphtheria toxin, ricin and venom phospholipase.

44. The monoclonal antibody of claim 1, 5, or 39, conjugated to a glycosidic enzyme.

45. A method for detecting adenocarcinoma, which comprises: contacting the monoclonal antibody of claim 2, 3, or 39 with a human tissue or fluid sample, and detecting the interaction of said antibody with any antigenically-corresponding adenocarcinoma cells or antigenic determinants thereof in said sample.

46. The method of claim 45, wherein the human tissue is breast tissue.

47. The method of claim 45, wherein the human tissue is ovarian tissue.

48. The method of claim 45, wherein the fluid sample is lymph, serum or pleural fluid.

49. A method for detecting poorly-differentiated breast carcinoma tumors, which comprises: contacting the monoclonal antibody of claim 7 or 39 with human breast tissue, and detecting the interaction of said antibody with any antigenically-corresponding breast carcinoma cells.

50. A method for differentiating adenocarcinomas from normal tissue of ovarian, uterine, colonic, pancreatic or prostatic histotypes, which comprises:

contacting the monoclonal antibody of claim 10 or 39 with human ovarian, uterine, colonic, pancreatic or prostatic tissue and detecting the interaction of said antibody with any antigenically-corresponding adenocarcinoma cells.

51. The method of claim 45, wherein the interaction is detected by immunohistological staining.

52. The method of claim 50, wherein the interaction is detected by immunohistological staining.

53. A method for localizing human adenocarcinomas in vivo, which comprises:

a) purifying the monoclonal antibody of claim 3 or 39;

b) radiolabelling said antibody;

c) administering said antibody to a human patient in a suitable carrier;

d) localizing the monoclonal antibody by external scintigraphy, emission tomography or radionuclear scanning.

54. A method for passive immunotherapy for the treatment of adenocarcinoma, which comprises: a) purifying the monoclonal antibody of claim 1 or 39; and b) administering said monoclonal antibody to a human adenocarcinoma patient in a suitable carrier.

55. A method of passive immunotherapy for the shrinkage of solid breast tumors, which comrpises: a) purifying the monoclonal antibody of claim 1 or 39; and b) administering said monoclonal to a human breast carcinoma patient in a suitable carrier.

56. A method of immunotherapy for the treatment of adenocarcinoma, which comrpises: a) purifying the monoclonal antibody of claim 40; and b) administering said monoclonal to a human adenocarcinoma patient in a suitable carrier.